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(57) Abstract

Process for increasing the rate of production of carbon dioxide, ethanol and other fermentation products such as citric acid, produced by yeast such as *Saccaromyces cerevisiae* during fermentation and decreasing biomass production by regulating the rate of glycolysis indirectly through changing the energy balance of the cell, i.e. by reducing intracellular ATP levels. Modifications for so altering the glycolysis rate involve the use of either a regulated ATP hydrolysis within the cell or a regulated leakage of ATP from the cell. This invention encompasses several ways for altering the yeast ATP level including (a) engaging futile metabolic cycles to increase ATP consumption; (b) using an altered exocellular acid phosphatase so that it becomes intracellular to increase intracellular ATP hydrolysis; (c) using a drug which uncouples the plasma membrane ATPase thereby consuming an abnormally high level of ATP; and (d) using a drug which allows the release of ATP from the cell to lower the intracellular ATP level. This invention further encompasses regulating the genetic modifications described in greater detail herein. By these means, such modifications may be turned off during growth of the yeast on a commercial scale, and then turned on before or during, and preferably before or at a very early stage of, the dough-rising phase.

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YEAST STRAINS

Field of the Invention

This invention relates to yeast, especially bakers or brewers yeast having higher rates of carbon dioxide and ethanol production. For example, a higher rate of carbon dioxide evolution provides more rapid leavening for bakers yeast or a higher rate of ethanol production provides reduced brewing time for brewers yeast. This invention also relates to a novel means for regulating gene expression involving the regulated removal of a transcriptional block.

Background of the Invention

Yeasts are one of the oldest cultivated plants. Their use dates back to about 2000 B.C. Among the various recognized yeast genera, Saccharomyces is of the greatest economic and practical importance, as it is used extensively in the baking, brewing and winemaking industries, as well as in the production of biomass. See, for example, Reed, G., "Yeasts" in the Kirk-Othmer Encyclopedia of Chemical Technology, 24:771-806 (John Wiley & Sons, New York, 1984).

The major, although not the only, function of yeast in fermentation is to provide a source of carbon dioxide and ethanol. Sufficient yeast must be added to dough, wort or other fermentable substrate to obtain the desired rate of carbon dioxide and ethanol production. If a more active yeast were available, less yeast could be used, at a corresponding savings in cost.

Improving the fermentative power of yeast is an ongoing research effort. Both the dried yeast and the moist yeast forms may be improved to increase their carbon dioxide producing ability so as to (1) reduce fermentation time and/or (2) enable the use of less yeast, a considerable cost factor in baking as well as brewing. Improvements in yeast fermentative power also makes the preparation of the more stable active dry yeast (ADY) form attractive. Generally, upon preparation of the ADY from a fresh yeast culture, about 40% of the fermentative ability is lost. Methods for eliminating or reducing this problem are continuously being sought.

One approach to improve dried yeast activity involves a modification of either the drying process, or the drying properties of the yeast strain, so as to prevent the loss of activity which occurs during drying. Process improvements have been made, and classical genetic approaches have been applied to this problem, with moderate success. See for example, U.S. Pat. 3,993,783.

Another approach to solving the problem of low activity dry yeast is described in U.S. Pat. 4,420, 563. Yeast having improved leavening activity, particularly in sweet doughs of high sugar content, was produced by the incremental addition of salts to the growing yeast culture during the latter propagative stages.

The present invention is directed to genetic and chemically induced modifications which increase the carbon dioxide and ethanol producing activity of any yeast strain.

Summary of the Invention

Disclosed herein are processes for increasing the rate of production of carbon dioxide, ethanol, and other fermentation products (eg, citric acid) produced by yeast. Generally, the processes involve reducing the level of ATP in the yeast cell, thereby stimulating glycolysis. In one aspect of the invention the ATP level is reduced by substituting in the yeast genotype (eg, via a single copy or multicopy vector or via cointegration into the yeast genome) a regulatable promoter for the natural promoter of a gene encoding a metabolic enzyme, thus permitting the regulatable expression of the enzyme, thereby permitting the metabolic reaction catalyzed by the enzyme to proceed at the same time as the reverse reaction such that ATP is consumed. In another aspect of the invention, the modification also involved inserting into the yeast genotype a gene encoding a metabolic enzyme, but in this aspect, under the expression control of a promoter permitting constitutive expression of the gene, thereby permitting the metabolic reaction catalyzed by the enzyme to proceed at the same time as the reverse reaction such that ATP is consumed. Further modifications of this invention involve modifying the gene encoding the metabolic enzyme, eg, to prevent or eliminate allosteric or other inhibition or inactivation of the enzyme.

In one embodiment, the enzyme is FDPase. The FDPase gene may additionally be mutagenized such that the codon for Ser-12 of the enzyme is replaced with a codon for an amino acid other than serine or such that allosteric inhibition, eg by AMP and/or fructose-2,6-diphosphate, is reduced or eliminated. In another embodiment, the genetic modification involves modifying a gene for an exocellular

APase, eg by removing that portion of the gene encoding the leader sequence, such that the modified enzyme remains within the yeast cytoplasm and catalyzes the hydrolysis of intracellular ATP.

One advantage provided by this invention is that the genetic modifications may be "turned on" only during, and preferably at the early stage of, the leavening phase and not during the production-level growth of the yeast. This is a significant advantage in baking yeasts. Alternatively, the genetic modifications may be constitutively expressed such that they are turned on during large scale production, ie commercial scale growth of the yeast, for the enhanced production of fermentation products such as ethanol.

Regulation of the genetic modifications may be achieved by using a temperature sensitive promoter or a promoter which is induced by the presence of a specific substance such that the enzyme is expressed only at a predetermined temperature or in the presence of the substance. Alternatively, expression control may be provided by inserting into the yeast genome a FLP gene construct described in greater detail herein.

Vectors useful in these processes include single copy, centromere containing plasmids and multicopy plasmids containing the yeast 2u origin of replication, as well as vectors permitting the cointegration thereof into the yeast genome.

This invention further encompasses processes in which the ATP level is reduced by growing or contacting the yeast with a chemical capable of directly or indirectly inducing ATP consumption. Such chemicals include relatively small organic molecules as well as proteins such as yeast killer toxin.

This invention further encompasses genetically modified yeasts produced by the methods disclosed herein.

Finally, this invention also encompasses a method and vector system for the regulated expression of heterologous genes in yeast, bacteria, and higher eucaryotic cells such as plant and mammalian cells.

Brief Description of the Drawings

Figure 1 illustrates the construction of an integrating yeast plasmid containing the FLP gene expressed from the Gal1 promoter.

Figure 2 illustrates a yeast plasmid containing the Gal promoter fused to the beta-gal coding region from E. coli.

Figure 3 illustrates the induction of ß-galactosidase activity from the Gal1 promoter under glucose limited growth.

Figure 4 illustrates the loss of a heterologous gene by regulated site specific recombination.

Figure 5 illustrates the loss of a transcriptional block from the GPDH promoter expressing the yeast FDPase gene.

Figure 6 illustrates a plasmid containing an expression block bounded by Sal1/BglII restriction sites.

Figure 7 illustrates the construction of an expression vector containing origins of replication, and selectable markers for both yeast and E. coli .

Figure 8 sets forth the nucleotide sequence of the cloned yeast FDPase gene.

Figure 9A and B set forth the deduced amino acid sequence of the cloned FDPase enzyme and the amino acid sequence of pig FDPase, respectively.

Figure 10 illustrates the construction of a FDPase cassette for expression from a heterologous promoter.

Figure 11 illustrates the synthesis of an expression vector where FDPase is expressed from the GPDH promoter.

Figure 12 illustrates the construction of an amino terminal deletion of FDPase.

Figure 13 illustrates the exchange of the serine residue at the protein kinase recognition site for an alanine.

Figure 14 illustrates carbon dioxide evolution during fermentation of yeast cultures expressing wild type FDPase or amino terminally deleted FDPase.

Figure 15 illustrates carbon dioxide evolution during fermentation of yeast cultures expressing FDPase lacking the recognition site for cyclic AMP dependent protein kinase.

Figure 16 illustrates the apparatus used in the gassing test.

Figure 17 illustrates the improvement in gassing power of a strain of yeast expressing the gene for the non-phosphorylated FDPase enzyme.

Figure 18 illustrates the introduction of the yeast acid phosphatase promoter into the yeast expression vector.

Figure 19 illustrates the DNA sequence for the yeast acid phosphatase promoter and the beginning of the structural gene for acid phosphatse (PHO 5).

Figure 20 illustrates the introduction of a unique Bgl II site at the 3' end of the acid phosphatase promoter.

Figure 21 illustrates the introduction of a restriction site into the acid phosphatase gene for expression of the mature protein.

Figure 22 illustrates the synthesis of an expression vector which expresses the mature acid phosphatase gene from the acid phosphatase promoter.

Figure 23 illustrates the introduction of a yeast centromere into the plasmid containing the modified acid phosphatase gene.

Detailed Description

The glycolytic pathway of yeast has been studied extensively for many years. See for example, Fraenkel, "Carbohydrate Metabolism" in The Molecular Biology of the Yeast Saccharomyces; Metabolism and Gene Expression, Cold Spring Harbor Laboratory, New York. The glycolytic fermentation of sugars is a very inefficient process from the viewpoint of both energy and biomass conversion. Nonetheless, yeasts can grow more rapidly by anaerobic glycolysis, where energy is derived from substrate level phosphorylation, than they can by the oxidative phosphorylation of aerobic growth.

In the course of the research leading to this invention, we have found that the rate of glycolysis is regulated by the yeast cellular levels of adenosine triphosphate (ATP). This discovery is indeed surprising since allthough a regulatory role of ATP in some enzymatic steps has been observed ,to our knowledge a regulatory role for ATP in the overall glycolytic pathway has never before been demonstrated or even suggested, despite the extensive study of glycolysis heretofore. The present invention makes use of this discovery by providing modifications for decreasing the cellular levels of cytoplasmic ATP, thus stimulating glycolysis, and thereby increasing the rate of evolution of carbon dioxide and ethanol by the yeast.

As mentioned above, baker's yeast may be improved if its carbon dioxide producing ability during leavening can be increased. Brewers yeast may also be improved by increasing the rate of fermentation (i.e. alcohol production) and reducing the time required for the brewing process. The reduced biomass production during

fermentation processes using yeast modified in accord with this invention also improved the fermentation process for ethanol production. This invention provides modifications for making these improvements by regulating the rate of glycolysis through abnormal reduction of the cellular ATP level. By "abnormal reduction of the cellular ATP level" we mean simply a reduction induced by a method of this invention.

Since the enzymes involved in glycolysis are normally in excess, the rate of glycolysis may be limited by the allosteric inhibition of certain glycolytic enzymes by cellular metabolites, including intracellular adenosine triphosphate (ATP) levels, as discovered in the course of the research described hereinafter. Thus, reducing cellular ATP levels in accord with this invention stimulates glycolysis, thereby increasing the rate of carbon dioxide and ethanol output and decreasing biomass production.

In the case of bakers yeast, however, if this alteration of the yeast were to operate during normal growth the yeast would be very inefficient at producing biomass. Thus it would be commercially impractical to grow the yeast due to increased production costs. It is therefore desirable that the genetic modifications of this invention be regulated for baker's yeast production so that it only operates during the leavening (or fermentation) process, not during the production (growth phase) of the yeast itself. One significant advantage of this invention is the ability to turn on the genetic modification and attendant increase in carbon dioxide production such that the increased production accrues at the beginning or early stages of the dough-rising phase. This restraint is not necessary for brewing or

ethanol-producing yeasts, however, where production is simultaneously obtained during fermentation.

Several approaches and specific examples thereof for reducing cytoplasmic ATP levels are described below. Generally, these approaches include (i) establishing a regulated futile metabolic cycle which consumes ATP, (ii) introducing or enhancing cytoplasmic ATPase activity in a regulated manner, and (iii) reducing cytoplasmic ATP levels by affecting plasma membrane function. As will become clear from the description which follows, approaches (i) and (ii) above involve introducing an altered gene or altered gene function into the yeast strain via recombinant DNA techniques. Before describing the above-mentioned approaches in further detail, it may be helpful to first describe the tools for effecting such genetic modifications, i.e., suitable vectors, regulated promoters and methods for introducing these changes into baking or brewing strains of yeast.

Vectors

The gene responsible for inducing a futile cycle or the cytoplasmic acid phosphatase gene may be cloned under the transcriptional control of a promoter into two types of autonomously replicating expression vector: a single copy, centromere containing plasmid, or a multicopy plasmid. Alternatively the DNA may be introduced into the yeast chromosome by recombination. In addition these vectors contained a selection gene and 3' noncoding regions, as are well known in the art.

The vectors are transformed into a strain of yeast and the yeast cells selected for those containing the vector by a selection protocol as is well known in the art. The selected yeast cells containing the vector, i.e. transformed cells, are grown in a suitable growth media and the promoter induced to start the loss of cytoplasmic ATP.

Suitable selection genes are well known in the art. It is preferred that the selection agent be one that prevents cell growth in the absence of the selection gene. Thus, cells that lose the plasmid in large scale culture do not contain the selection gene and will not over-grow during the fermentation. However, it may be desirable in the commercial production of desired products to avoid the use of certain cell toxins, thereby simplifying the product purification steps. Thus, a desirable selection gene is one that enables transformants to grow in a media lacking a nutrient required for growth of the parental strain. Useful selection genes in the practice of this invention include for example, URA3, LEU2, etc.

The vectors useful herein can be synthesized by techniques well known to those skilled in the art. The components of the vectors such as selection genes, promoters, and the like can be obtained from natural sources or synthesized as discussed below. Basically, components which are available in large quantity (i.e., which are present on natural plasmids, e.g. the 2u plasmid of yeast, or which can be readily synthesized) can be assembled with the appropriate use of restriction enzymes and T4 DNA ligase. If a component is not available in large quantity, it can be amplified by insertion into a bacterial cloning vector such as a plasmid or phage as is well known in the art. Then, with appropriate use of restriction enzymes, large

quantities of vector can be obtained by techniques well known in the art by simply culturing the bacteria, digesting its DNA with an appropriate endonuclease, separating the DNA fragments, identifying the DNA containing the component of interest and recovering same. Ordinarily, a transformation vector is assembled in small quantity and then ligated to a suitable autonomously replicating synthesis vector such as a plasmid or phage for production of larger amounts of transformation vector DNA. The well known pBR322 plasmid, for example, can be used in most cases.

The synthesis vectors are used to clone the ligated transformation vectors in conventional fashion, e.g. by transformation of a permissive prokaryotic organism, replication of the synthesis vector to high copy number, and recovery of the synthesis vector by cell lysis and separation of the synthesis vector from cell debris. The resulting harvest of synthesis vector can be directly transformed into yeast cells. Many different types of yeast vectors are readily available and may be substituted where appropriate (Parent et al., Yeast 1:83-138 (1985).

Certain vectors including transformation vectors and vectors containing various elements such as specific promoters, the FLP gene and an FDPase gene have been deposited on November 5,1986 in E. coli HB101 with the American Type Culture Collection,12301 Parklawn Drive,Rockville, MD 20852(USA), including the following:

1. AZ402 plasmid contains URA3 transcriptional block flanked by FLP recognition sequences within a Bgl II/Sal I cassette (ATCC No. 67257)

2. AU125 plasmid contains an FDPase gene operatively linked to the GPDH promoter (ATCC No.67256)

3. AT823 plasmid contains an FDPase gene (ATCC No.67258)

4. AR900 plasmid contains the FLP gene operatively linked to the Gal I promoter with restriction sites for the substitution of other promoters for the gall promoter; plasmid provides for regulated expression of the FLP gene (ATCC No. 67259)

5. YOp1 plasmid is an illustrative example of a selectable 2u plasmid (ATCC No. 67260)

6. BA601 plasmid contains mutagenized (ser-12 to ala) FDPase gene operatively linked to the GPDH promoter (ATCC No. 67261)

7. M138 multicopy plasmid for expression of a cytoplasmic (mutagenized) APase gene (ATCC No.67262)

8. N305 similar to M138 except that the plasmid contains a yeast centromere and is therefore a single copy plasmid (ATCC No. 67263)

Regulated Promoters

Numerous promoters useful in yeast transformation vectors are known in the art which may be used in the practice of this invention. As discussed in greater detail herein, regulated promoters, many of which are known in the art, are preferred in certain embodiments of this invention. Examples of regulated yeast promoters are those from galactose , maltose, phosphate or nitrogen metabolism, isocytochromes and alcohol dehydrogenase II. A specific example of a regulated promoter is that from the yeast acid phosphatase gene (PHO5). The promoter reportedly acts in response to levels of inorganic phosphate in the media. It is possible that a strong promoter such as that from acid phosphatase (APase) may yeild a higher than optimal expression level for certain embodiments of this invention, eg.futile cycling. The desired regulated promoter can be modulated in several ways. For example, a cloned copy of the acid phosphatase promoter can be mutated in vitro using the method of Shortle (Shortle et al., Proc. Natl. Acad. Sci. USA, 79:1588 (1982)) or small deletion/substitutions within the promoter can be generated by insertion of linkers by known techniques (McKnight and Kingsbury, Science 217:316 (1982), Heffron and McCarthy, Proc. Natl. Acad. Sci. USA 75:6012 (1978)). A pool of DNA fragments containing the mutated acid phosphatase promoter is inserted into a yeast/E. coli shuttle plasmid where the promoter expresses a detectable marker, for example, the beta-galactosidase or beta-lactamase gene from E. coli (Guarente and Ptashne, Proc. Natl. Acad. Sci. USA, 78:2199 (1981); Rose et al., Proc. Natl. Acad. Sci. USA, 78:2460 (1981); Martinez and Casadaban, Mol. Cell. Biol., 3:580 (1983); and Silverman et al., Mol. Cell. Biol.,

2:1212 (1982)). Transformed yeast colonies are then screened for the production of the detectable marker, e.g. on media containing 5-bromo-4-chloro-3-indolyl-beta-D-Glactoside (X-gal) where the desired phenotype gives white colonies on high phosphate media and light blue colonies on low phosphate media. Both strong and weak promoters may thus be identified. DNA is obtained from the yeast cells showing a suitable phenotype and transformed into E. coli using standard techniques. Ampicillin resistant colonies must contain the yeast plasmid. Plasmid DNA is made from these E. coli transformants and the acid phosphatase promoter from the plasmids used for expression.

Alternatively, a temperature sensitive regulatory gene may be used. For example, many mutations in the pho R and pho U regulatory genes of the acid phosphatase pathway are found to give constitutive expression at 36°C and normal regulation at 23°C (Ueda et al., J. Bact., 122:911 (1975)). The plasmid of interest containing the PHO5 promoter is transformed into a yeast strain containing a pho R or pho U temperature sensitive mutation and the acid phosphatase promoter is regulated by changing the culture temperature in a high phosphate containing medium. This mode of regulation is also used in conjunction with a weak (mutated) acid phosphatase promoter.

Another method for regulating the modifications of this invention to use another regulated promoter which is naturally much weaker than the acid phosphatase promoter. Several such promoters have been identified. For example, the promoter from the yeast HO gene. The expression of this promoter is controlled indirectly by mutations

in the Sir locus (Rine, "Regulation and Transposition of Cryptic Mating type genes in Saccharomyces cerevisiae", Ph.D. thesis, University of Oregon at Eugene (1979)). In a normal "wild type" cell which has Mat a at the Mating type locus, an alpha cassette HML and the HO allele at the homothallic locus, the HO promoter would be turned on. If the strain carries a Sir mutation however both Mat a and Mat alpha (from HML) are expressed and the HO promoter would be turned off. Therefore a strain carrying a temperature sensitive Sir mutation may be used where the HO promoter is expressed at low temperature but repressed at high temperature.

The regulated promoter is turned "off" during growth of baker's yeast and "on" at the end of fermentation, or in the bread dough, by changing the yeast culture conditions. For example, when using the APase promoter the yeast are grown in the presence of a regulated amount of phosphate so that the culture uses all of the phosphate before the end of fermentation. At this time, the APase promoter is induced by the depletion of phosphate from the fermenter. If a temperature sensitive expression system is used, the culture temperature is set such that the promoter is turned "off" during growth and "on" at the end of fermentation.

Regulated site specific recombination as a mechanism for expression of a futile cycle

One problem for optimizing the expression of an ATP reducing process in baking yeast is the difficulty in identifying a promoter which can be regulated so that it is turned off during growth of the yeast but on during leavening. This is especially difficult since the

yeast is used for many different types of baking applications where the consistency of the yeast may not be controllable due to different handling conditions which may affect the regulated promoter. If a promoter regulated by the same growth conditions as is normally required for production of CO_2, i.e. glycolysis, is used the yeast should be as consistent as the standard baking yeast.

Unfortunately glycolytic genes are not strongly transcriptionally regulated nor are they continually suppressed during growth of the baking yeast. However, triggering the expression of an ATP reducing process by a glycolytic promoter at the end of the growth fermentation would overcome these objections.

A novel approach was therefore devised which allows the yeast to grow up without wasting ATP, but have the ATP reducing process expressed from a glycolytic promoter during the leavening process. This is accomplished using a regulated, site specific recombination event to remove a transcriptional block within the promoter, for example the GPDH promoter, and is described in more detail below. It should be noted that this unusual expression strategy may be used for regulating the expression of a wide variety of genes, including, but not limited to, those encoding enzymes for futile cycling or cytoplasmic phosphatases.

The 2 micron plasmid of yeast has been shown to undergo site specific recombination between two inverted repeats (Hartley and Donelson, (1980) Nature 286:860-864). This recombination is catalysed by a specific recombinase (FLP), whose gene is located on the 2 micron plasmid (Broach and Hicks (1980) Cell 21:501-508). The

GalI promoter is suppressed by glucose and induced by galactose (Yocum et al (1984) Mol. Cell. Biol. 4:1985-1998). Thus if the GalI promoter were used to regulate FLP expression in the baking yeast, providing the natural 2 micron plasmid had been lost, the FLP gene will not be expressed until galactose is present. A growth fermentor is normally run under glucose limitation to prevent the Crabtree effect and the formation of ethanol and to maximize the production of cells. We have found that the GalI promoter is not glucose repressed under these conditions. Addition of galactose to the fermenter thus induces the Gall promoter with the consequent expression of the FLP gene. curing the 2 micron plasmid of yeast may be accomplished using, for example, the method of Erhart and Hollenberg, J. Bact. 156:625-635. The FLP gene could also be expressed by another regulateable promoter as described above.

A clone of the FLP gene was therefore isolated and expressed from the regulated GalI promoter. To this end, the 2 micron plasmid of yeast was digested with XbaI and then digested with HindIII and a 1,450 bp XbaI/HindIII fragment isolated by preparative gel electrophoresis. The isolated fragment was then inserted into a conventional yeast plasmid containing the GalI promoter at the PvuII/SphI sites such that the FLP fragment was expressed from the GalI promoter to produce plasmid AR900 (Fig 1). This allowed expression of the FLP gene protein from the Gall promoter.

Regulation of the Gal1 promoter during continuous growth under glucose limitation.

To test further the GalI promoter as a mechanism for regulating the FLP gene, a plasmid (PRY171 Fig 2) containing the Gal promoter fused to the β-galactosidase coding region from E. coli (Parent et al supra), was transformed by integration into the genome of a laboratory yeast strain, e.g. KY114. This strain was innoculated into a chemostat where the culture was grown under glucose limitation in yeast minimal media. The culture was stabilized by growth, with a doubling time of 3.5 hours, for 48 hours. Galactose was added to the fermentor at 2% final concentration and samples taken at regular intervals. β-galactosidase activity and protein concentration were measured using standard techniques (Miller 1972 Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; Rose et al., 1981 Proc. Natl. Acad. Sci. 78:2460-2464). As can be seen (Fig. 3) the GalI promoter induced beta-galactosidase activity under conditions of glucose-limited growth when galactose was present.

To test the feasibility of using this galactose inducible FLP gene to regulate recombination, a strain of yeast lacking endogenous 2 micron plasmid and which had been previously transformed by integration with a plasmid containing a heterologous gene flanked by tandem repeats from the 2 micron plasmid of yeast (Hartley and Donelson 1980 Nature 286:860-864; Senecoff et al 1985 Proc. Natl. Acad. sci. USA 82:7270-7274; Andrews et al (1985) Cell 40:795-803) was transformed with a plasmid containing the Gal/FLP fusion gene.

If the Gal/FLP fusion works, the strain should express the heterologous gene when grown on glucose (which supresses the GalI promoter). When the strain is grown on galactose, however, the heterologous gene should be excised from the genome by recombination resulting in loss of the gene (Fig. 4). This was indeed found to be the case since on glucose media 97% of the colonies expressed the heterologous gene while on galactose media 0% of the colonies contained the heterologous gene activity. Next an expression block, e.g. a DNA sequence containing a transcriptional block such as the URA3 HindIII fragment, or a silencer region or a transcription terminator (Brand et al., 1985, Cell 21:501-508), is inserted into the promoter, e.g. the GPDH promoter, between the upstream activation sequence and the translational start site. This transcriptional block element is flanked by DNA sequences (inserted as synthetic oligonucleotides, illustrated in Table 1) shown to be recognized by the FLP gene product as substrates for site-specific recombination (Senecoff et al supra ;Andrews et al supra) . Thus regulation operates by addition of galactose to a growing nonglucose-repressed culture of yeast. Galactose induces the synthesis of the FLP protein which catalyzes a recombination event between the DNA sequences flanking the expression block. The recombination event removes the expression block, thereby allowing expression of the heterologous gene, e.g. the gene for FDPase from the GPDH promoter. Fig. 5.

Such a block surounded by recombination sites illustrated in Fig 6 has been deposited and can be inserted into the promoter of choice by those skilled in the art by first inserting a BglII/SalI linker into the promoter sequence, at a site which allows expression, using site

TABLE 1

Sequence for FLP Catalysed Site Specific Recombination Site

TCGACGCTTTGAAGTTCCTATTCCGAAGTTCCTA
GCGAAACTTCAAGGATAAGGCTTCAAGGAT (cont'd on next line)

TTCTCTAGAAAGTATAGGAACTTCAGAGCGCTTA
AAGAGATCTTTCATATCCTTGAAGTCTCGCGAATCTAG

directed mutagenesis as described (Zoller and Smith N A R 10:6487-6500 (1982); Methods Enzymol. 100:468-500 (1983), DNA 3:479-488(1984), and then inserting the transcriptional block from plasmid AZ402 as a BglII/SalI fragment.

This FLP expression system can be used to regulate the expression of virtually any gene in a variety of host cells. Generally, the regulated expression in a host cell of a heterologous DNA sequence may be effected using a two vector system. The first vector contains a DNA sequence encoding a FLP protein (FLP DNA) operatively linked to a regulatable promoter. An example of such a vector is AR900 which contains the FLP gene operatively linked to the Gal I promoter. Similar vectors containing other promoters may be constructed by routine methods by excising the Gal I promoter from AR900 with Eco RI and Sph I and substituting therefor any desired promoter, again using conventional techniques and appropriate linkers, if necessary. The second vector contains the heterologous DNA sequence to be expressed, operatively linked to a promoter which contains, inserted therein, an expression block flanked by DNA sequences (flanking DNA) which are recognized by FLP protein. Such vectors may be constructed by conventional

means using the Sal I - Bgl II casette from AZ402 together with readily available and/or synthesized components. The Sal I - Bgl II cassette from AZ402 contains the URA3 expression block as a Hind III fragment flanked by FLP-recognized recombination sites. Other expression blocks may be substituted for URA3 using conventional methods, and again, conventional linkers, if necessary. The expression block, e.g. the Sal I - Bgl II cassette from AZ402, is then inserted, with conventional linkers if necessary, into the promoter region of a vector containing the heterologous DNA sequence operatively linked to a promoter such that expression is blocked. Suitable insertion may be readily confirmed empirically by observation of the phenotype of cells transformed with the vector and/or by monitoring the culture medium for the absence of the expression product. Transformation of a host cell with both the first and second vectors described above yields an expression system wherein the FLP protein produced by expression of the FLP DNA in the first vector catalyzes a recombination between the flanking DNA of the second vector, thereby removing the expression block and allowing expression of the heterologous DNA sequence. Naturally, when using any specific host cell, the vectors should contain any genetic elements required by the particular host, as is well known in the art.

Using the above-described vectors and regulated promoters we have produced yeast strains characterized by higher rates of CO_2 production, and have produced such strains by introducing into the host strain (i) an ATP-consuming futile cycle and, in another embodiment of the invention, (ii) enhanced cytoplasmic ATPase activity.

Genetic Modification of Baking and Brewing Strains

Baking and brewing yeast strains present a more difficult substrate for transformation than laboratory strains since they are

polyploid and do not generally contain auxotrophic markers which can be used for the selection procedures which are well known in the art.

However a modified or a heterologous gene/promoter construct such as the FDPase gene linked to the GPDH promoter, discussed below, can be introduced into the strain of yeast used for baking by using dominant drug resistant genes such as the antifungal agents gentamycin (G418) (Jiminez and Davies, Nature 287:869 (1980))or hygromycin B (Gritz and Davies, Gene 25:179-188 (1983)) . As an example of this approach, a resistance gene coding for aminocyclitol phosphotransferase (ACPT) is carried by the bacterial transposon TN601 which confers resistance to G418 , but its promoter is weak and therefore is only partially effective at conferring resistance. Jimenez and Davies, supra. The promoter is exchanged for a yeast promoter (e.g., the yeast glyceraldehyde phosphate dehydrogenase promoter) . A plasmid is then constructed containing the desired gene/promoter construct with its natural chromosomal flanking regions together with the TN601 ACPT described above. This plasmid does not contain a yeast origin of replication. The strain of bakers yeast is transformed with this plasmid and transformants selected on G418 plates. The plasmid copy of ACPT can only be stably maintained if the plasmid is integrated into the yeast genome at the natural gene locus. This results in a tandem duplication of the gene (e.g. FDPase) separated by the plasmid and ACPT sequences. Growth of these transformants in the absence of G418 allows for the loss of the plasmid by "looping out" leaving behind either the "wild type" or the introduced sequence. These G418 sensitive clones are then screened by Southern hybridization of genomic DNA using oligonucleotide probes for the presence of the heterologous construct using standard techniques.

(i) ATP-consuming Futile Cycles

One genetic modification for reducing cellular ATP levels is the use of a normal metabolic pathway in an abnormal manner to consume ATP, so that glycolysis is stimulated. Most preferably, the chosen pathway is cyclic, so that its abnormal use results in no significant net accumulation or depletion of any required metabolic intermediate, substrate or product. Many metabolic pathways in yeast are capable of running in opposite directions depending on the growth conditions or requirements of the cell. For example, metabolite "A" may be converted into metabolite "B", or "B" into "A", as required by the cell. Causing such a pathway to run in both directions at the same time results in no net accumulation or loss of metabolites but does, however, consume the energy required to run the pathways, and in this respect is a "futile" cycle. Of course, other futile cycles involving additional steps can also be used (e.g. A$\longrightarrow$ B$\longrightarrow$ C$\longrightarrow$ A). Transcriptional, translational and post-translational controls can be used to turn on or off such a futile cycle, where, for every revolution of the cycle, one molecule of ATP is consumed without producing any net accumulation of the product or loss of substrate. In bakers yeast, the metabolic change necessary to reduce ATP levels and thereby stimulate glycolysis is preferably regulated so that it is operable only during leavening.

A preferred futile cycle for consuming ATP is: fructose-6-phosphate$\longrightarrow$ fructose-1,6-diphosphate$\longrightarrow$ fructose-6-phosphate. The enzymes involved in this pathway, phosphofructo-

kinase and fructose 1,6-diphosphatase (FDPase or FBPase) have been extensively characterized (Bloxham and Lardy, The Enzymes, Vol. 8, Boyer, Ph.D., Ed., pp 239-278 (1973); Uyeda, Adv. Enzymology, 48, 193-244, (1979); Foy and Bhattachargee, Arch. Microbiol., 129, 215-220 (1981); Funayama et.al. (1979)). By cloning the gene for FDPase and exchanging its promoter for one which is regulated, the gene is expressed at will. We have found that expressing this gene during growth on glucose is sufficient to accomplish a considerable loss of ATP and a consequent increase in the rate of glycolysis. Optimizing the FDPase-driven futile cycle requires understanding the natural regulation of FDPase. For the sake of clarity, a brief discussion of FDPase regulation is provided.

FDPase Regulation

The regulation of FDPase has been studied by a number of researchers. In order to prevent futile cycling between the synthesis and hydrolysis of fructose-1,6-diphosphate in wild type yeast this enzyme is rapidly inactivated when glucose is added to cells growing on non-fermentable carbon sources. This inactivation of FDPase appears to occur in three stages. An initial inhibition of enzyme activity is accomplished by allosteric regulation (Lenz and Holzer, F.E.B.S. Lett., 109, 271-274 (1980); Wolf and Holzer, Transport and Utilization of Aminoacids, Peptides and Proteins by Microorganisms, Payne, J.W., Ed., John Wiley, Chinchester, 1980; Tortora et.al., Biochem. Biophys. Res. Comm.. 100 688-695 (1981)). When glucose is added to yeast cells, the concentration of fructose-2,6-diphosphate rises within seconds from undetectable levels to concentrations of

several uM, enough to partially inhibit FDPase. The mechanisms that regulate the synthesis of fructose-2,6-diphosphate are unclear (Gancedo et al., J. Biol. Chem, 258 5998-5999 (1983)). After the initial rapid inhibition, a second step involving phosphorylation of the FDPase occurs over a period of several minutes (Muller and Holzer, Biochem. Biophys. Res. Comm., 103 926-933 (1981); Tortora et.al., supra; Purman et al., Biochem. Biophys. Res. Comm., 107 1482-1489 (1982)). The state of phosphorylation of FDPase is controlled by a specific kinase and a specific phosphatase. The phosphorylation occurs at a particular serine residue (Muller and Holzer supra; Mazon et al., J. Biol. Chem., 257 1128-1130 (1982)). The modified FDPase is less active than the unmodified enzyme. Finally, the phosphorylated enzyme appears to be a substrate for a specific protease which catalyzes an irreversible inactivation of the FDPase, over a period of about an hour.

Genetic Modifications for Regulating FDPase

There are several genetic approaches that are applicable to block this inactivation of FDPase. Mutants which do not synthesize fructose-2,6-diphosphate, or which have an FDPase that does not bind the inhibitor, block the inactivation cascade at the beginning. Site specific mutagenesis of the serine that otherwise becomes phosphorylated yields an enzyme resistant to the second and third steps. Some enzyme activity remains after the initial partial inhibition by fructose-2,6-diphosphate (Lenz and Holzer, supra; Wolf and Holzer, supra; Tortora et al., supra). This enzyme activity is enough to cause significant futile cycling.

We have identified the site of phosphorylation (Ser_{12}) in FDPase since it is the only consensus recognition site for cAMP dependent protein kinases present in the cloned FDPase gene ($Arg_9.Arg_{10}.Asp_{11}.Ser_{12}$) and have readily altered it by conventional site specific mutagenesis. (Zoller and Smith, supra). We have found that an amino acid substitution for the serine to prevent phosphorylation is sufficient to produce enough enzyme activity to cause a significant level of futile cycling. However the enzyme is also inhibited by high concentrations of AMP (Taketa and Pogell, J. Biol. Chem., 240 651-662 (1965)). For further optimization the enzyme (which is already somewhat resistant to AMP inhibition by virtue of the substutition at Ser_{12}) may additionally be altered at its AMP binding site to overcome this inhibition. The binding site of AMP on the enzyme has been characterized. To achieve enhanced enzymatic activity for the futile cycle this site may be mutated in vitro and reintroduced into yeast and the loss of inhibition by AMP indirectly assayed. On plates a mutant form of the enzyme no longer inhibited by AMP allows the yeast to grow normally on gluconeogenic carbon source but very poorly on glucose, thus permitting a convenient assay for such a modification.

Since the enzymes involved in this futile cycle are fairly major yeast proteins, this pathway is sufficient to consume a considerable amount of ATP. The stimulation of glycolysis is "fine tuned" to give any desired level of carbon dioxide output by changing the copy number of the altered fructose diphosphatase gene, the strength of the promoter or the promoter used in the FDPase or FDPase-variant expression vector as described.

Alternative Futile Cycles

There are a surprisingly large number of alternative futile cycles which could be engaged to consume ATP. For example, the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase can be reversed (Katz, J. and Rognstad, R., Cur. Top. Cell. Reg., 10 237-289 (1976) and Reeves, R.E., Biochem. J. 125 531 (1971)). Alternatively many other futile cycles will waste ATP including those found in amino acid biosynthesis and degradation, polyphosphate synthesis and degradation, fatty acid biosynthesis and pyrimidine biosynthesis. Since all are strictly controlled at the transcriptional level, a futile cycle may be induced by changing the regulation of the enzymes involved by changing their promoters. However, such cycles have additional regulatory mechanisms. Generally, the additional regulation involves feedback inhibition or allosteric modulation of enzyme function by intermediates or energy metabolites. If desired, such allosteric binding sites may be modified to eliminate or reduce allosteric inhibition in analogous fashion to the methods described herein in the illustrative case of FDPase. Other classes of regulation involve the sequestering of one of the enzymes in an organelle, where substrate availability can be controlled, or in the trapping of unstable intermediates in an enzyme complex, allowing them to be quickly converted to a stable intermediate. All of these pathways are potential inducible ATP hydrolysing processes and may therefore be used to consume ATP via appropriate genetic modification.

Where desired the quantity of ATP consumed by the modified cell may be regulated to maximize CO_2 and ethanol production. This can

be accomplished by using a stronger or weaker promoter or modulating its activity, or by using a temperature sensitive regulatory gene whose degree of regulation is dependent upon the temperature of the culture as described above.

(ii) Introduction of Enhanced Cytoplasmic Acid Phosphatase Activity

An alternative modification for regulating the rate of glycolysis involves producing a cytoplasmic acid phosphatase to hydrolyze organic phosphates including ATP. The normally exocellular acid phosphatase of the yeast Saccharomyces cerevisiae is an inducible non-specific phosphatase located in the periplasm. The gene for this enzyme has been recently cloned and characterized. (See Rogers et al., Proc. Nat'l. Acad. Sci., U.S.A., 79 2157-2161 (1982)). Preventing the phosphatase from being secreted into the periplasm of the cell (e.g., by genetic modification removing the enzyme's secretory leader) will result in dephosphorylating organic phosphates in the cytoplasm, including ATP. However, this non-specific phosphatase will also dephosphorylate other important organic phosphates causing serious damage to the metabolism of the cell. The level of "trapped" cytoplasmic phosphatase must therefore be strictly controlled. For example it was found that the natural promoter for yeast acid phosphatase (PHO5) expreses too much ATPase activity when fully induced to achieve an increase in the rate of glycolysis. To have a regulated method of hydrolysing ATP by a cytoplasmic acid phosphatase, a weaker promoter is preferably used such that it has the desired effect when fully induced or the basal level of an

inducealbe promoter can be used as illustrated below. This can be accomplished in several ways, examples of which have been described above.

iii Modification of Plasma Membrane Function

Another embodiment of this invention involves the plasma membrane ATPase, which uses over one third of the ATP generated by fermentation in order to regulate the concentration of ions in the cytoplasm. This is the major pathway for ATP hydrolysis in the cell, Stimulation of this pathway may thus be used to reduce the cellular levels of ATP and thereby stimulate fermentation (i.e. ethanol and carbon dioxide production).

There is some evidence that some drugs which affect the plasma-membrane proton pump induce the cell to stimulate fermentation (Serrano, Eur. J. Biochem., 105:419 (1980)). The stimulation found is very low. The significance of this finding appears to have been overlooked and would be discounted as within experimental error without further work. The reasons for this stimulation are not completely clear since other drugs (i.e. dicyclohexylcarbodiimide and diethylstilbestrol), which also disrupt the plasma membrane proton gradient do not cause a stimulation of fermentation. One explanation, supported by this work, is that dicyclohexylcarbodiimide and diethylstilbestrol inhibit the plasma-membrane ATPase and therefore do not reduce cellular ATP levels. Considerable damage to the general metabolism of the cell may also be inflicted by disruption of other ATP dependent membrane functions. Dinitrophenol appears to act by directly disrupting the proton gradient across the plasma

membrane. This stimulates the plasma membrane ATPase and may thereby reduce the levels of cellular ATP.

An alternative method of stimulating the plasma membrane ATPase would be by the use of small proteins such as ribonuclease (Alper et al (1967) J. Bact 93:759-765; Yphantis et al (1967) J. Bact 94:1509-1515) or yeast killer toxin (Bussey and Sherman (1973) Biochimica et Biophysica Acta 298:868-875).

The invention will be further illustrated with reference to the following Examples, which are purely exemplary, and should not be taken as limiting the true scope of the present invention, which is set forth in the claims.

EXPERIMENTAL EXAMPLES

Materials

All DNA restriction and metabolism enzymes were purchased from New England Biolabs. These enzymes were used in conditions and buffers described by New England Biolabs, except mung bean exonuclease which was obtained from PL Biochemicals and used as described. ATP and the deoxynucleoside triphosphate (dNTP's), i.e. dATP, dGTP, dCTP and dTTP, were purchased from PL Biochemicals and [^{32}P] was obtained from New England Nuclear Corporation.

Ligation reactions were carried out as described by Maniatis et.al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold spring Harbor, N.Y. (1982), the disclosure of which is incorporated herein by reference, using the buffer described at

page 246 thereof and using a DNA concentration of 1-100 ug/ml, at a temperature of 23°C for blunt ended DNA and 16°C for "sticky ended" DNA. Electrophoresis was done in 0.5-1.5% agarose gels containing 90 mM Tris-borate, 10 mM EDTA.

After DNA digestion restriction enzymes were inactivated by heating to 65°C for 10 minutes. When performing sequential reactions the DNA was precipitated with 70% ethanol after each step. After "filling in" a restriction site by reaction with the large fragment of DNA polymerase (Klenow) and the four dNTP's, the reaction mixture was made 10mM magnesium chloride and an equal volume of 5M ammonium acetate was added. The DNA was precipitated with 2 volumes of ethanol at -20° and the DNA pelleted by centrifugation at 4°C for 10 minutes in an Eppendorf microfuge. The ethanol was poured off and the pellet dissolved in 10ul/ug DNA of 0.2M sodium acetate. The DNA was re-precipitated with 2 volumes of ethanol and centrifuged as before. The DNA pellet was dried under vacuum before proceeding to the next step in the construction. Synthetic oligonucleotides were kinased as described in Maniatis et al., supra and annealed by heating to 65°C and slow cooling to 4°C before use.

DNA preparation and transformation

Purification of "super coiled" plasmid DNA from E. coli and the transformation of E. coli was as described in Maniatis et al, 1982, supra. Transformation of yeast was as described by Hinnen et al, Proc. Natl. Acad. Sci. USA, 75, pp 1929-33 (1978), except that 1.2M Sorbitol was used instead of 1.0M. Small scale plasmid preparation

for screening transformed bacteria was essentially that described (Maniatis et al., 1982, supra; Holmes and Quigley, Anal. Biochem. 14 p 193 (1981)) except that the RNAse digestion was performed after the restriction enzyme digestion by adding 1 ul of a 1 mg/ml solution of RNAse (Boehringer Mannheim) to the well of the agarose gel just before electrophoresis.

Strains and Media

E. coli strain HB101 was used for all bacterial transformations. Yeast strains DB745 (Botstein et al., Gene 8 pp. 17-24 (1979)), KY114 or ATCC 26675 were used. E. coli were grown in LB media with or without ampicillin (49 ug/ml) as described (Maniatis et al., 1982, supra). Prior to transformation, yeast were grown at 30°C in media containing 1% yeast extract (Difco), 1% Bacto Peptone (Difco) and 2% glucose. Yeast minimal media contained 5 gm ammonium sulfate, 10 gm glucose, 40 mg adenine, 60 mg leucine, 2ug biotin, 400 ug calcium pentothenate, 2 ug folic acid, 2 ug inositol, 400 ug niacin, 100 ug p-aminobenzoic acid, 400 ug pyridoxine hydrochloride, 500 ug boric acid, 40 ug copper sulphate, 100 ug potassium iodide, 200 ug sodium molybdate, 400 ug zinc sulfate, 500 mg magnesium sulphate, 100 mg sodium chloride, 100 mg calcium chloride and 1 gm (high phosphate media) or 10mg (low phosphate media) potassium phosphate (monobasic) per liter. For induction of the acid phosphatase promoter, cells were pregrown at 30°C on high phosphate yeast minimal media, washed free of phosphate, and transferred to low

phosphate yeast minimal media to resume growth at 30°C. Maximum induction occurred 8 to 12 hours after transfer.

For the dinitrophenol experiments the yeast strain used in this study was Fleishman's active dry yeast. The yeast was grown in CO containing 1% yeast extract (Difco), 1% Bacto peptone (Difco), 0.05M di-Potassium phosphate and titrated to pH 5.6 with citric acid. The media was made up in 10 liter batches in 15 liter carboys and autoclaved for 2 hours at 121°C. After cooling, sterile glucose was added to 2% (w/v). Antifoam was added every 24 hours to prevent foaming. Dinitrophenol was added directly to the 15 liter carboy of media just before connection of the media feed to the chemostat and to the culture vessel to make the same concentration in the media feed. Dinitrophenol was made up in stock solution in ethanol. Cells were grown up overnight in CO before innoculation into the chemostat. Chemostat culture were grown in a New Brunswick Scientific model F-2000. The culture vessel had been adapted by the addition of a side arm to give a working volume of one liter. Spent culture was allowed to flow out of the vessel by means of a submerged open tube to prevent loss of the antifoam which tended to stay on the surface of the culture.

The fermentor was run at 30°C and an agitation setting of 4. Nitrogen was continuously bubbled through the vessel at a rate of 500 cc /min and the off gas passed through a moisture trap of Dry-Rite and into a Perkin Elmer Mass Spectrometer gas analyzer for the measurement of carbon dioxide.

In continuous culture, media was fed at a rate of 250 ml/hr using a Pharmacia model M3 pump. When carbon dioxide

measurements were being taken, all settings, volumes and temperature were checked and adjusted if necessary. Media feed, temperature and agitation were found to be fairly stable, however, the nitrogen gas feed varied by as much as 10% over a four hour period. Therefore, the output from the gas analyzer was fed into a chart recorder and the rate of flow of nitrogen adjusted manually over a two hour period. The rate of flow of nitrogen and the level of carbon dioxide in the off gas was checked for stability over this period before taking measurements of cell density in the culture. The culture was therefore demonstrated to be stable within the limits of detection before measurements were taken. This was born out by the reproducibility of the data. Culture density was measured by dilution of the culture ten fold in water and reading the density in a Bausch and Lomb Model Spectronic 20 at 600 nm.

Vector Construction

To minimize the size of the expression plasmid and to reduce the number of restriction sites, a plasmid was constructed which contained the uracil 3 gene (URA3) as a selection gene and the 2u origin of replication. Alternatively, another yeast plasmid could be used such as YEp24 or YEp13 or equivalent (Parent et al., supra). Our plasmid was derived from YIp5 (Botstein et al., supra) with the addition of a HaeIII/HpaI fragment, containing the origin of replication from the 2u plasmid of yeast. The plasmid, YOp1 (Fig. 7), was constructed by introducing the 2u origin into the EcoR1 site of YIp5. Plasmid DNA from YEp24 (Botstein et al., supra) was cut with restriction enzymes HaeIII and Hpa1 and the DNA run on a

preparative 1.0% Agarose gel. The 1.4 kb fragment containing the 2u origin of replication was identified by comparison with the migration pattern of molecular weight marker fragments and electroeluted into a well cut into the agarose. The DNA fragment was purified by passing the buffer from the well over a DEAE Sephacel column (Maniatis et al, supra). Plasmid YIp5 was cut with EcoR1 and the "sticky ends" "filled in" using the Klenow fragment of DNA polymerase 1 and all four dNTP's. The HaeIII/Hpa1 fragment from YEp24 was ligated into the "filled in" EcoR1 site of YIp5 (Fig. 7). The ligation mixture was transformed into HB101 and the resulting ampicillin resistant colonies screened for the presence of the 2u origin fragment. Since a "filled in" EcoR1 site ligated to a HaeIII site re-creates the EcoR1 site the orientation of the fragment was determined by mapping the resulting EcoR1 site to restriction sties on the plasmid. A plasmid (YOp1) having the EcoR1 site proximal to the Pst1 site within the ampicillin resistant gene was used in subsequent constructions.

Example 1

Loss of ATP by futile cycling

Isolation for the gene for Fructose 1,6-diphosphatase

The gene for FDPase was isolated by complementation of a deletion mutant of FDPase in E. coli (strain DF657, CGSC number 6695). A plasmid library of "wild type" yeast genomic DNA in a pBR322 plasmid vector was transformed into DF657 by selection for antibiotic resistance and a plasmid carrying the yeast FDPase gene

identified by its ability to allow the bacteria to grow on a gluconeogenic carbon source. The yeast FDPase clone was sequenced using the dideoxynucleotide sequencing method of Sanger et al., Proc. Natl. Acad. Sci. USA 74: 5463-5467, 1977. Fig. 8. Comparison of the amino acid sequence of yeast FDPase derived from the DNA sequence showed greater than 50% homology with the amino acid sequence of purified pig FDPase (Marcus et al (1982) Proc. Natl. Acad. Sci. USA 79:7161-7165) (Fig 9) confirming the correct identification of the yeast clone.

Since a futile cycle must be carefully regulated to prevent premature wasteage of ATP the first adaption of the natural FDPase gene was to change its promoter for that of a sequence which could be regulated during the fermentation. A restriction site analysis of the DNA sequences of yeast FDPase identified a NdeI site very close to the start of the coding region (Fig. 10). In vitro mutagenesis was used to adapt the 5' end of the clone for expression from a heterologous yeast promoter by converting the NdeI site to an SphI site. The FDPase gene was first subcloned into pBR322 to create plasmid AR705, Fig. 10. Plasmid AR705 was digested with Nde1 and treated with mung bean exonuclease. An adapter containing four out of six base pairs of an Sph1 site at one end and a Xho1 overhang at the other end was ligated to the plasmid, digested with XhoI and the plasmid closed using T4 DNA ligase to generate plasmid AT823, Fig. 10. The ligation mix was transformed into bacteria and ampicillin resistant colonies screened for the presence of the Sph1 and XhoI sites.

The FDPase gene in plasmid AT823 was ligated to the promoter from the gene for glyceraldehyde phosphate dehydrogenase (GAP491) [Holland and Holland (1980), J. Biol. Chem 255:2596-2605] as follows. Plasmid O605 (Fig. 13), derived from plasmid M903 , was digested with KpnI, treated with the Klenow fragment of DNA PolI, and cut with HindIII. Plasmid AT823 was cut with SphI, treated with the Klenow fragment of DNA PolI, cut with HindIII, and the 3.8 kb SphI/HindIII fragment was isolated and ligated to the HindIII/KpnI-digested O605 to generate plasmid AU125. Expression of the FDPase gene in plasmid AU125 is now regulated by the GPDH promoter.

FDPase mutagenesis

The plasmid containing the FDPase with the heterologous promoter was transformed into yeast and transformants tested for expression of the FDPase clone. FDPase activity was detectable when cells were grown under inducing conditions on a gluconeogenic or glytolytic carbon source. When growing using fermentation, it would be expected that allosteric inhibitors and enzyme inactivation would reduce enzyme activity.

Since inactivation is mediated by phosphorylation of a serine residue, a change in the structural gene contemplated by this invention is the elimination of this phosphorylation site. The serine residue which is phosphorylated has been identified as residue 12 (the only cAMP dependent protein kinase recognition site in the sequence and from peptide mapping of purified phosphorylated enzyme

and amino acid sequencing of the phosphorylated peptide (Rittenhouse et al (1986) J. Biol. Chem. 261:3939-3943).

It had previously been found that by performing a mild trypsin digest on liver FDPase one could generate an amino terminal deletion which retained activity (Chatterjee et al., 1985). Mammalian and yeast FDPase show a very high level of conservation. An amino terminal deletion of the yeast enzyme was made and tested for activity.

Fructose 1, 6 diphosphatase (FDPase) was hooked up to the yeast GPDH promoter from the EcoRV restriction site present in the DNA sequence within the amino terminus of the gene (Figure 12, AX4). This gave an amino-terminal deletion, to residue 19, which missed the protein kinase recognition site.

In addition, serine (residue 12) was changed to an alanine using site directed mutagenesis (Zoller and Smith supra). This is a conservative change which would not be expected to affect the activity of the non-phosphorylated protein but would prevent phosphorylation of the enzyme. The serine could also have been changed to a threonine, valine or cysteine or another amino acid using site directed mutagenesis (Figure 13). This was achieved by cloning that part of the sequence around this residue into a single stranded DNA virus, M13. A synthetic oligonucleotide was made which hybridized to this region of the DNA but was a mismatch at the serine residue such that the sequence substitutes an alternate codon. A double stranded molecule was then made from this hybrid by the use of the Klenow fragment of DNA polymerase Pol1. The reaction is

conducted in the presence of all four deoxynucleotide triphosphates and DNA ligase. This hybrid double stranded DNA was then cloned into bacteria, replicated, re-isolated and re-transformed into bacteria to resolve the two strands. Half of the progeny contain the sequence for the serine, and half contain the substituted sequence for the alanine or other codon of choice. They were distinguished by hybridization to a short oligonucleotide (17 bp) complimentary to the substituted sequence. This substituted gene was then put back into the multicopy GPDH expression vector as described above and transformed into a laboratory strain of yeast e.g. KY114.

In order to measure the rate of glycolysis, cultures of yeast expressing clones of FDPase from the glyceraldehyde phosphate dehydrogenase (GPDH) promoter were examined in small one liter fermentors. Cultures were grown in batch and nitrogen was bubbled through the culture at 110 ml/min. Carbon dioxide and cell density were measured periodically.

Initially, cultures of yeast expressing the wild type enzyme (plasmid AU125) and the amino terminal deletion (plasmid AX4) were compared to a control plasmid containing a non-expressed FDPase (plasmid AU110) (Figure 14). The level of carbon dioxide produced by the cultures at the beginning of the growth cycle is very similar. However at the end of the growth cycle, substantially higher levels of CO_2 were produced. The increase in carbon dioxide output of the strain carrying the amino terminal FDPase deletion is less than that expressing wild type enzyme. However FDPase does not appear to be innactivated in this yeast strain and the amino terminal deletion has a

considerably lower specific activity than the "wild type" enzyme. One would therefor have expected that the wild type enzyme would give a greater stimulation of glycolysis than the enzyme containing the deletion. It is very intersting that carbon dioxide output increases only at the end of the growth cycle. This would suggest that FDPase is being regulated allosterically during exponential growth phase. The most likely candidates for this regulation are fructose 2, 6 diphosphate or AMP.

After specific point mutants in the phosphorylation site of FDPase had been created, fermentation experiments were repeated. In these experiments, strain ATCC 26675, expressing FDPase containing the serine to alanine mutation from the GPDH promoter (plasmid BA601) or a control plasmid where FDPase was not expressed (plasmid BA802) were tested. Strain ATCC 26675 has been found to give the highest level of inactivation of many yeast strains tested. These cultures should thus give a conservative estimate of the possible increase in gassing power.

Fermentations were performed as before and carbon dioxide and cell density measured. Carbon dioxide output is again increased toward the end of the growth cycle in the strain expressing FDPase (Figure 16). The fermentation experiment was repeated and gave good reproductibility. Cultures were harvested at the end of the growth cycle, centrifuged, and examined using a gassing test described below.

Gassing tests were performed in an apparatus illustrated in Figure 16 . The flask contained on gram of cells, one gram of

glucose and 10mls of media (yeast nitrogen base, Difco) and had a final OD 600 nm of 12. All solutions were at 32°C. The water bath was also at 32°C. Tube a was open during the first 10 minutes after the flask was placed in the water bath. Measurements of CO_2 evolution were made periodically by closing tube a and measuring the amount of CO_2 evolved in burette X after adjusting the level of liguid in burette Y to that in burette X to bring the gas in burrette X to atmospheric pressure. Measurements were taken until 100 ml of CO_2 had been produced. The rate of CO_2 production in the strain containing the plasmids described above are illustrated in Figure 17. In this test the strains expressing the mutant FDPase gave an increase of 25% in gassing power.

In addition to the previously described modifications, this invention further contemplates alteration of the allosteric regulation of FDPase by fructose 2,6-diphosphate or AMP.

Fructose-2,6-phosphate is synthesized via an enzymatic pathway from fructose-6-phosphate by the enzyme fructose-6-phosphate-2-kinase (Clifton and Fraenkel, J. Biol. Chem., 258:9245 (1983) and Pilkis et al., J. Biol. Chem., 259:949 (1949)).

One method for reducing inhibition of enzyme activity is to mutate the cloned copy of FDPase in vitro (see e.g., Shortle et al., Proc. Natl. Acad. Sci., 79:1588 (1982)) and introduce it back into the cell on a self-replicating selectable yeast plasmid followed by assaying for the loss of the inhibitory effects of fructose 2,6-diphosphate and AMP. In principal, the loss of a site where an allosteric inhibitor binds is often a fairly conservative change in the

enzyme structure since even a slight modification of the binding site is expected to greatly alter its affinity for fructose-2,6-diphosphate. This approach requires a good assay for the altered enzyme. Since FDPase is under the control of an inducible promoter, when the futile cycle is working efficiently, under inducing conditions, mutant colonies growing on a fermentable carbon source are very small but under non-inducing condition the colonies are normal in size. The suspected mutant colonies are also plated on a gluconeogenic carbon source where they grow normally under inducing conditions. Such colony screening methods may therefore be used to assay for the altered enzyme.

Finally, the altered FDPase is introduced into the strain of yeast used for baking by the procedures described above. Bakers yeast containing the altered FDPase are found to have substantially increased leavening ability.

Example 2

Expression of a cytoplasmic acid phosphatase

Preparation of the Promoter Fragment of APase

Plasmid YIpAPII (Rogers etal 1982, supra) containing a full copy of the large subunit, P60 (PHO 5), of the acid phosphatase enzyme was mapped with various restrictions enzymes and HpaII was found to give a 700 bp restriction fragment containing 600 bp of upstream DNA sequence from the initiator (or start) codon for the structural gene whose position is known by reference to the ClaI site on the fragment (Thill et al, Molecular Cell Biology 3 pp 570-9 (1983)). Plasmid

YIpAPII was cut with HpaII and the DNA run on a preparative 1.5% agarose gel. The band of 700 bp, containing the promoter was electroeluted into a well cut into the gel as before and purifed on a DEAE Sephacel column. The fragment was mixed with YOpl cut with ClaI and the DNA ligated with T4 DNA ligase. Since HpaII and ClaI have self complementary "sticky ends" these DNA's will ligate together. The ligation mix was transformed into HB101 and the ampicillin colonies screened for the presence of the promoter fragment. One such plasmid, 920, (see Fig.18) was used for further constructions.

From the DNA sequence of the fragment of the acid phosphatase (PHO 5) gene Fig. 3 (Thill et al, 1983, supra; Arima et al, N.A.R. 11 pp 1657-72 (1983), an area having four out of the six bases of a BglII restriction endonuclease recognition site was identified 5bp upstream from the initiator ATG. This area was used to create a BglII site at this point in the APase promoter sequence using a synthetic oligonucleotide linker of self complementary sequence CTAGCATGCTAG.

Plasmid 920 was cut with KpnI (see Fig. 20) and the DNA treated with the double strand exonuclease Bal31 (Legerski et al, N.A.R. 5 pp 1145-1463 (1978)). At set time intervals, the reaction was stopped by the addition of ethylenediaminetetraacetic acid (EDTA) to 0.05M (Legerski et al, (1978) supra). A portion of the plasmid from each time interval was digested with ClaI and run out on a 12% poly-acrylamide gel. The time point where the 300 bp ClaI/KpnI fragment had been digested to approximately 270 bp was noted. The

remainder of the Bal31 treated plasmid from this time point was treated with the Klenow fragment of DNA Polymerase I and the four dNTP's. A linker of self complementary sequence CTAGCATGCTAG was kinased, annealed and ligated to this plasmid DNA. The DNA was then circularized with T4 DNA ligase and transformed into HB101. Approximately two thousand ampicillin resistant colonies were washed from the plates and supercoiled plasmid DNA made from these E. coli cells (Maniatis et al, 1982, supra). This pooled plasmid DNA was cut with the restriction enzyme BglII and the DNA run out on a preparative agarose gel. DNA running as a cut linear band was eluted into a well cut into the agarose and purified on a DEAE sephacel column. This purified DNA was re-circularized with T4 DNA ligase and transformed into HB101. Ampicillin resistant colonies were screened for the presence of a BglII site. The only way for the plasmid to obtain a BglII site was for the site to be created at the junction of the Bal31 digested DNA and the linker. The only available sequence where this could occur within several hundred bp upstream of the Kpn1 site is 5b in front of the ATG initiation codon. One such plasmid containing a BglII site, D718 (Fig. 20) was checked and shown to be as expected by the dideoxynucleotide sequencing method of Sanger (Sanger et al, Proc. Natl. Acad. Sci. 74 pp 5463-67 (1977)).

Plasmid YIpAP11, has been deposited with the American Type Culture Collection in E. coli HB101 as follows:

E coli HB101 (YIpAP11) - ATCC No. 39570

Creation of a restriction site at the leader/native protein junction

From the sequence of acid phosphatase gene it can be seen (Fig. 18) that there is a Kpn1 site close to the start of the mature sequence. This has enabled us to introduce a restriction site at the junction of the leader sequence and the mature protein sequence using a synthetic oligonucleotide of sequence GCTCGAGGTAC / CGAGCTC.

Since there are several Kpn1 sites in the acid phosphatase gene a fragment had to be subcloned from the 5' end of the gene. Plasmid YIpAP11 was cut with BamH1 and Sal1 and the fragment containing the promoter subcloned into the BamH1/Sal1 sites of YOp1. (Fig. 21) Transformed bacteria were screened and plasmid 801 was found to have the correct sequence. To introduce a restriction site at the 5' end of the mature sequence plasmid 801 was cut with Kpn1 and the adapter described above ligated to the Kpn1 site (Fig 21). The plasmid was then cut with BamH1 and the site "filled in" with the Klenow fragment of DNA polymerase 1 and the plasmid re-circularized. Transformed bacterial colonies were screened for the presence of the adapter. One such plasmid (J401) was used for further constructions. As can be seen from Fig 21 the adapter creates a Xho1 site at the junction of the leader and mature acid phosphatase such that if the plasmid is cut with Xho1 and the overhang digested with mung bean exonuclease there is a blunt site created at the correct position in the sequence at the start of the mature coding region.

Synthesis of a "leader less" clone of acid phosphatase

First the full length copy of acid phosphatase was constructed from plasmid J401. Plasmid YIpAP11 was cut with BamH1 , the 5' overhang "filled in" with the Klenow fragment of DNA polymerase 1 and then cut with Sal1 and the BamH1/Sal1 fragment containing the APase gene purified by preparative gel electrophoresis. This fragment was then ligated into the Sal1/Nru1 sites of plasmid J401 (Fig 22). A "filled in" BamH1 site ligated to an Nru1 site recreates the BamH1 site. Next the acid phosphatase promoter was reattached to the structural gene. Plasmid D718 was cut with BglII and an adapter of sequence GATCACCAATG which recreates the acid phosphatase
TGGTTAC

promoter sequence to the initiator methionine codon, ligated to the BglII site . The plasmid was then cut with EcoR1 and the EcoR1 to BglII adapter fragment (Fig 22) cloned into plasmid K219 which had been cut with Xho1, treated with mung bean exonuclease to flush the ends of the DNA and then cut with EcoR1. Transformants were screened and plasmids containing the correct restriction fragments were sequenced using the dideoxynucleotide sequencing method. Plasmid M138 was found to have the correct sequence at the junction of the promoter and the mature gene.

Addition of a yeast centromere to the plasmid

The acid phosphatase promoter is inducible about 1,000 fold. The copy number of a yeast plasmid may be varied by using different origins of replication or a yeast centromere (Clark & Carbon, 1980;

Tschumper & Carbon, 1983). A yeast centromere lowers the copy number of a 2u origin plasmid to about 1 copy per cell.

Plasmid M138 was cut with EcoR1 and the ends blunted with the Klenow fragment of DNA polymerase 1. Next, plasmid YCP19 (Fig. 23), which contains the centromere from chromosome 4 of yeast (Parent et al, supra), was cut with HindIII and the ends blunted with the Klenow fragment of DNA polymerase 1. The fragment containing the centromere was then ligated into the EcoR1 site of M138 to produce plasmid N305 (Fig 22).

Expression of cytoplasmic acid phosphatase

Plasmids M138 and N305 were transformed into yeast together with a control plasmid M721. Uracil prototrophs were selected and grown in high phosphate MO media containing an excess of glucose(4%), in a 1 liter New Brunswick Scientific model F-200 fermenter. During growth, cell density was measured using a Bausch and Lomb Model Spectronic 20 at 600 nm. The results are set forth in Tables 1 and 2 below. The fermenter was run at 30°C and an agitation setting of 4. Nitrogen was continuously bubbled through the vessel at a rate of 430 cc/min and the off gas passed through a moisture trap of Dry-Rite and into a Perkin Elmer Mass Spectrometer gas analyzer to measure CO_2.

TABLE 2

CARBON DIOXIDE EVOLUTION IN UNINDUCED CULTURES

CONTROL PLASMID M721

Time Minutes	Cell Number (x106)	% Carbon Dioxide
0	0.25	0.08
80	0.64	0.21
150	0.90	0.16
215	1.35	0.19
280	1.8	0.26
340	2.3	0.34
405	3.3	0.45
470	4.75	0.61
520	6.25	0.73
580	7.0	0.79
630	8.5	0.85
690	10.0	0.85
710	11.0	0.75

TABLE 2 CON'T

PLASMID N305

Time Minutes	Cell Number (x106)	% Carbon Dioxide
0	1.1	0.17
60	1.35	0.22
108	1.5	0.26
165	2.0	0.35
245	3.0	0.50
345	5.0	0.76
390	6.5	0.92
465	8.5	1.05
520	10.0	1.15
610	14.5	1.02
685	16.0	0.86

TABLE 2 CON'T

PLASMID M138

Time Minutes	Cell Number (x106)	% Carbon Dioxide
0	2.7	0.72
48	3.5	0.77
77	4.0	0.87
107	5.0	1.02
165	7.2	1.31
200	8.0	1.56
238	8.5	1.70
286	10.5	1.75
363	13.0	1.75
450	16.5	1.65
568	20.0	1.60
632	21.0	1.44

The level of carbon dioxide produced by the strains carrying the three different plasmids during growth on high phosphate media was found to vary (table 1). Then the basal level of promoter activity in high phosphate media is sufficient to produce an effect on the rate of glycolysis. When the data was compiled and normalized for the same stage in the growth cycle, it was noted that the level of carbon dioxide produced by cultures growing in high phosphate media increased with the copy number of the plasmid. The strain carrying

the multicopy plasmid produced twice as much carbon dioxide as the control and the strain carrying the single copy plasmid produced an intermediate level. Thus, the level of acid phosphatase can be controlled thereby controlling the level of cytoplasmic ATP in accord with this invention and increasing the rate of production of carbon dioxide.

Example 3

The effect of Plasma Membrane Uncouplers on the Rate of Glycolysis

When increasing amounts 2,4-dinitrophenol were added to stable chemostat cultures of Fleishman's baking yeast a considerable stimulation in carbon dioxide production is observed.

Initial experiments were performed to determine the basal level of carbon dioxide per cell under the growth conditions defined by the chemostat and to determine the stability of the culture. An overnight culture was innoculated into the chemostat and grown up for twenty four hours as a batch culture. Media feed was started and the culture left to stabilize for a further twenty-four hours before measurements were taken. Carbon dioxide production and cell number were monitored over a forty-eight hour period. These results are shown in Table 3. Time is given in hours after media addition start-up. Carbon dioxide is given as a percentage in the gas stream.

Table 3 Chemostat Stability

Time after startup Hours	Culture Density O.D. 600 nm	Cell Number Cells/ml x 10^{-8}	Carbon Dioxide in the off gas %
26	0.33	1.1	2.4
29.5	0.32	1.1	2.3
32	0.31	1.0	2.3
34	0.33	1.1	2.2
50	0.31	1.0	2.3

The culture appeared to be relatively stable.

Dinitrophenol was added to the media feed at 5 uM. Dinitrophenol was also added to the culture vessel at 5 uM as given in Experimental Procedures.

The culture was left to stabilize for forty-eight hours. As can be seen from Table 4, 5 uM dinitrophenol had little effect on carbon dioxide production.

Table 4 The Effect of Dinitrophenol On The Rate of Fermentation

Concentration of Dinitrophenol uM	Culture Density cells/ml x 10^{-8}	Carbon Dioxide in the off gas %	Relative Carbon Dioxide Production per cell
0	1.1	2.2	1.0
5	1.1	2.3	1.0
20	0.75	1.85	1.2
50	0.75	2.0	1.3
100	0.67	2.0	1.5
200	0.55	1.9	1.7
0	1.1	2.3	1.0

The concentration of dinitrophenol in the culture was slowly increased stepwise to 200 uM. After each step addition of dinitrophenol, the culture was left to stabilize for thirty-six to forty-eight hours before measuring cell density and carbon dioxide concentration. As shown in Table 4, above, with increasing concentration of dinitrophenol there was an increase in the level of carbon dioxide produced per cell.

If the effect of dinitrophenol on cellular metabolism had been the uncoupling of the plasma membrane ATPase one would have expected an increase in carbon dioxide output and a corresponding decrease in the cell density. It should be remembered that the dissipation of the plasma membrane ion gradient would also affect other cellular processes dependent on this ion gradient i.e. transport. It would not, therefore, be surprising to find that the metabolic efficiency of

the cell had been reduced by this treatment. This level of stimulation of glycolysis would therefore be expected to be a minimum.

The experiments have demonstrated that carbon dioxide production can be stimulated in a dose dependent way by dinitrophenol. The level of stimulation was found to be highly reproducible and gave a greater than two-fold increase in carbon dioxide production at the highest concentration used.

What is claimed is:

1. A process for increasing the rate of carbon dioxide and ethanol production of yeast, said process comprising reducing the level of ATP within the cell, thereby stimulating glycolysis.

2. A process of claim 1, wherein the ATP level is reduced by substituting in the yeast genotype a regulatable promoter for the natural promoter of a gene encoding a metabolic enzyme, permitting the regulatable expression of said enzyme, thereby permitting the metabolic reaction catalyzed by said enzyme to proceed at the same time as the reverse reaction such that ATP is consumed.

3. A process of claim 2 which further comprises genetically modifying the gene encoding the metabolic enzyme to prevent or eliminate allosteric or other inhibition or inactivation of said enzyme.

4. A process of claim 2 wherein said enzyme is fructose-1,6-disphosphate (FDPase).

5. A process of claim 4 wherein the gene encoding FDPase is mutagenized such that codon 12 of the mutagenized gene encodes an amino acid other than serine.

6. A process of claim 5 wherein the gene encoding FDPase is further mutagenized such that allosteric inhibition of the enzyme by fructose-2,6-diphosphate is reduced.

7. A process of claim 5 wherein the gene encoding FDPase is further mutagenized such that allosteric inhibition of the enzyme by adenosine monophosphate is reduced.

8. A process of claim 2 wherein the gene encoding the enzyme is not expressed during the production phase of yeast growth.

9. A process of claim 2 wherein the regulatable promoter is a temperature sensitive promoter such that the gene encoding the enzyme is expressed only at a predetermined temperature.

10. A process of claim 2 wherein the expression is under the control of a regulated genetic recombination catalyzed by FLP protein.

11. A process of claim 2 wherein the ATP level is reduced by genetically modifying a gene for an exocellular acid phosphatase (Apase) such that the APase remains within the yeast cytoplasm.

12. A process of claim 11 wherein the genetic modification comprises inserting into the yeast a vector containing a gene encoding a mature APase without a functional secretory leader sequence.

13. The process of claim 12 wherein the vector comprises an autonomously replicating single copy, centromere containing plasmid.

14. The process of claim 12 wherein the vector comprises a multicopy plasmid containing the yeast 2u origin or replication.

15. The process of claim 12 wherein the vector is inserted into the genome of the yeast.

16. A process of claim 1 wherein the ATP level is reduced by inserting into the yeast genotype a gene encoding a metabolic enzyme under the expression control of a promoter permitting constitutive expression of the gene, thereby permitting the metabolic reaction catalyzed by said enzyme to proceed at the same time as the reverse reaction such that ATP is consumed.

17. A process of claim 16 wherein the expression is further under the control of a regulated genetic recombination catalyzed by FLP protein.

18. A process of claim 1 wherein the ATP level is reduced by treating the yeast with a chemical capable of directly or indirectly inducing the consumption of ATP.

19. An improved yeast wherein the improvement comprises a genetic modification that reduces the level of ATP within the cell, thereby stimulating glycolysis and providing a yeast having high fermentation activity.

20. The yeast of claim 19 wherein the modification comprises the presence within the yeast of vector DNA containing an APase gene lacking a functional secretory leader sequence such that the APase expressed therefrom is not secreted from the cell.

21. The yeast of claim 19 wherein the modification comprises the presence within the yeast of vector DNA containing the gene for a metabolic enzyme under the expression control of a regulatable promoter permitting the regulated expression of said enzyme, thereby permitting the metabolic reaction catalyzed by said enzyme to proceed at the same time as the reverse reaction such that ATP is consumed.

22. The yeast of claim 19 wherein the modification comprises the presence within the yeast of vector DNA containing the gene for a metabolic enzyme under the expression control of a promoter permitting the constitutive expression of said enzyme, thereby permitting the metabolic reactin catalyzed by said enzyme to proceed at the same time as the reverse reaction such that ATP is consumed.

23. The yeast of claim 22 wherein the expression is further under the control of a regulated genetic recombination catalyzed by FLP protein.

24. A vector system for the regulated expression in a host cell of a heterologous DNA sequence which comprises:

(a) a first vector containing a DNA sequence encoding a FLP protein (FLP DNA) operatively linked to a regulatable promoter, and

(b) a second vector containing the heterologous DNA sequence operatively linked to a promoter, which promoter contains inserted therein, an expression block flanked by DNA sequences (flanking DNA) which are recognized by FLP protein,

such that FLP protein produced by expression of the FLP DNA in the first vector catalyzes a recombination between the flanking DNA, thereby removing the expression block and allowing the expression of the heterologous DNA sequence.

AMENDED CLAIMS
[received by the International Bureau on 21 April 1987 (21.04.87); original claims 1-24 replaced by amended claims 1-30 (7 pages)]

1. A process for increasing the rate of carbon dioxide and ethanol production of yeast, said process comprising regulating a reduction of the level of ATP within the cell such that the rate of leavening is increased.

2. A process for increasing the rate of carbon dioxide and ethanol production of yeast, said process comprising reducing the level of ATP within the cell such that the rate of brewing is increased.

3. A process for increasing the rate of carbon dioxide and ethanol production of yeast, said process comprising reducing the level of ATP within the cell such that the rate of fermentation is increased.

4. A process of claim 1 wherein the ATP level is reduced by substituting in the yeast genotype a regulatable promoter for the natural promoter of a gene encoding a metabolic enzyme, permitting the regulatable expression of said enzyme, thereby permitting the metabolic reaction catalyzed by said enzyme to proceed at the same time as the reverse reaction such that ATP is consumed.

5. A process of claim 4 which further comprises genetically modifying the gene encoding the metabolic enzyme to prevent or eliminate allosteric or other inhibition or inactivation of said enzyme.

6. A process of claim 4 wherein said enzyme is fructose-1, 6-diphosphate (FDPase).

7. A process of claim 6 wherein the gene encoding FDPase is mutagenized such that codon 12 of the mutagenized gene encodes an amino acid other than serine.

8. A process of claim 7 wherein the gene encoding FDPase is further mutagenized such that allosteric inhibition of the enzyme by fructose-2, 6-diphosphate is reduced.

9. A process of claim 7 wherein the gene encoding FDPase is further mutagenized such that allosteric inhibition of the enzyme by adenosine monophosphate is reduced.

10. A process of claim 4 wherein the gene encoding the enzyme is not expressed during the production phase of yeast growth.

11. A process of claim 4 wherein the regulatable promoter is a temperature sensitive promoter such that the gene encoding the enzyme is expressed only at a predetermined temperature.

12. A process of claim 4 wherein the expression is under the control of a regulated genetic recombination catalyzed by FLP protein.

13. A process of claim 1, 2 or 3 wherein the ATP level is reduced by genetically modifying a gene for an exocellular acid phosphatase (APase) such that the APase remains within the yeast cytoplasm.

14. A process of claim 13 wherein the genetic modification comprises inserting into the yeast a vector containing a gene encoding a mature APase without a functional secretory leader sequence.

15. The process of claim 14 wherein the vector comprises an autonomously replicating single copy, centromere containing plasmid.

16. The process of claim 14 wherein the vector comprises a multicopy plasmid containing the yeast 2u origin of replication.

17. The process of claim 14 wherein the vector is inserted into the genome of the yeast.

18. A process of claim 2 or 3 wherein the ATP level is reduced by inserting into the yeast genotype a gene encoding a metabolic enzyme under the expression control of a promoter permitting constitutive expression of the gene, thereby permitting the metabolic reaction catalyzed by said enzyme to proceed at the same time as the reverse reaction such that ATP is consumed.

19. A process of claim 18 wherein the expression is further under control of a regulated genetic recombinantion catalyzed by FLP protein.

20. A process of claim 1, 2 or 3 wherein the ATP level is reduced by treating the yeast with a chemical capable of directly or indirectly inducing the consumption of ATP.

21. An improved yeast wherein the improvement comprises a genetic modification that reduces the level of ATP within the cell, thereby providing a yeast having high fermentation activity.

22. An improved yeast wherein the improvement comprises a genome modification that regulates a reduction of the level of ATP within the cell thereby providing a yeast having an increased rate of leaving activity.

23. An improved yeast wherrein the improvement comprises a genetic modification that reduces the level of ATP within the cell thereby providing a yeast having high brewing activity.

24. The yeast of claim 21, 22 or 23 wherein the modification comprises the presence within the yeast of vector DNA containing an APase gene lacking a functional secretory leader sequence such that the APase expressed therefrom is not secreted from the cell.

25. The yeast of claim 22 wherein the modification comprises the presence within the yeast of vector DNA containing the gene for a metabolic enzyme under the expression control of a regulatable promoter permitting the regulated expression of said enzyme, thereby permitting the metabolic reaction catalyzed by said enzyme to proceed at the same time as the reverse reaction such that ATP is consumed.

26. The yeast of claim 21 or 23 wherein the modification comprises the presence within the yeast of vector DNA containing the gene for a metabolic enzyme under the expression control of a promoter permitting the constitutive expression of said enzyme, thereby permitting the metabolic reactive catalyzed by said enzyme to proceed at the same time as the reverse reaction such that ATP is consumed.

27. The yeast of claim 22 wherein the expression is further under the control of a regulated genetic recombination catalyzed by FLP protein.

28. A vector system for the regulated expression in a host cell of a heterologous DNA sequence which comprises:

(a) a first vector containing a DNA sequence encoding a FLP protein (FLP DNA) operatively linked to a regulatable promoter, and

(b) a second vector containing the heterologous DNA sequence operatively linked to a promoter, which promoter contains inserted therein, an expression block flanked by DNA sequences (flanking DNA) which are recognized by FLP protein, such that FLP protein produced by expression of the FLP DNA in the first vector catalyzes a recombination between the flanking DNA, thereby removing the expression block and allowing the expression of the heterologous DNA sequence.

29. A method for regulating expression in a host cell of a heterologous DNA sequence which comprises:

(a) transfecting said host cell with

(i) a first vector containing a DNA sequence encoding a FLP protein (FLP DNA) operatively linked to a regulatable promoter, and

(ii) a second vector containing the heterologous DNA sequence operatively linked to a promoter, which promoter contains inserted therein, an expression block flanked by DNA sequences, (flanking DNA) which are recognized by FLP protein,

(b) culturing said transfected host cell to express FLP protein of said first vector catalyzing a recombination between the flanking DNA, thereby removing the expression block and allowing the expression of the heterologous DNA sequence.

30. A cDNA encoding a peptide sequence substantially as set forth in Figure 8 or a DNA sequence which hybridizes with the DNA sequence of Figure 8.

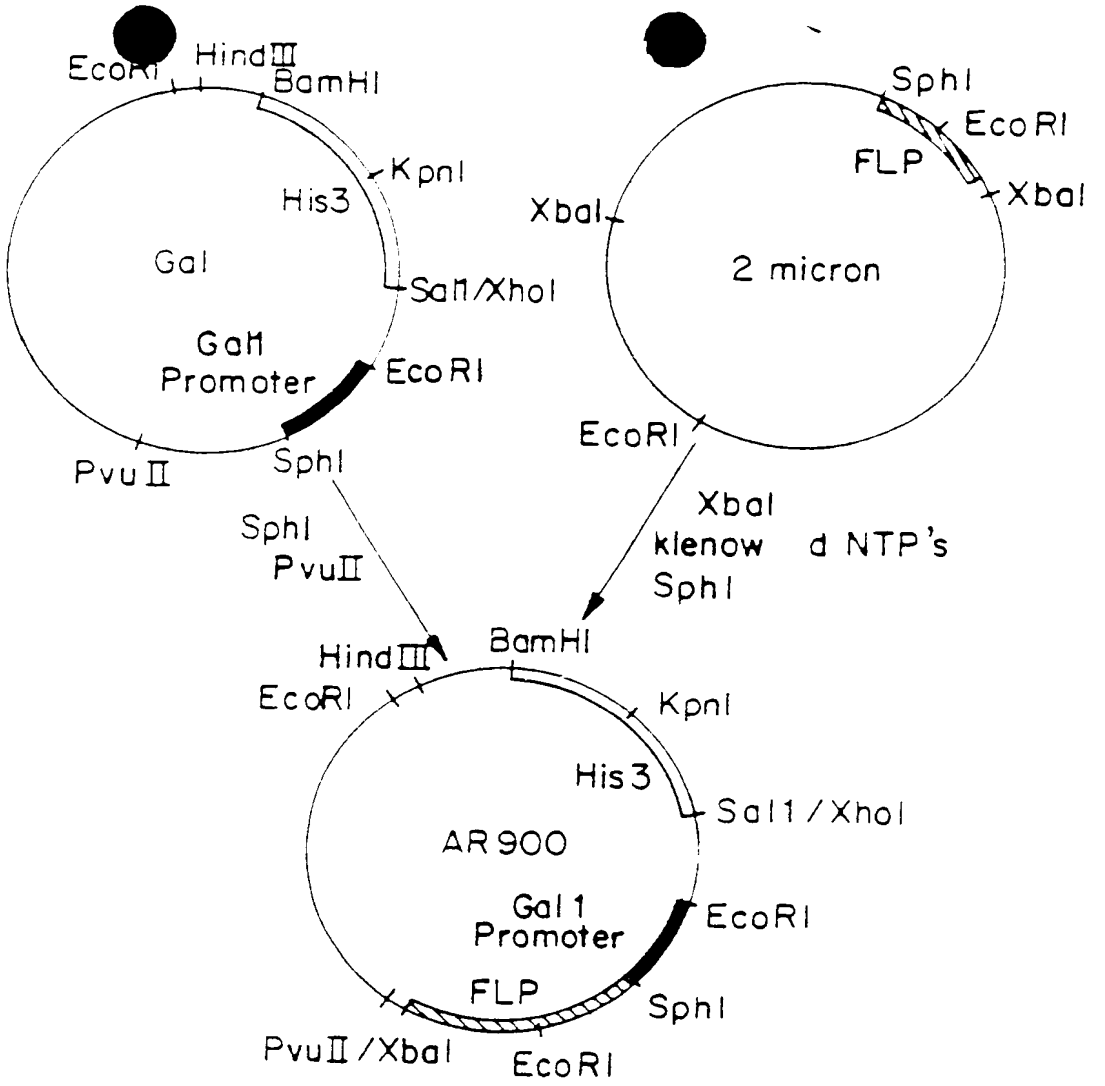

FIG. 1

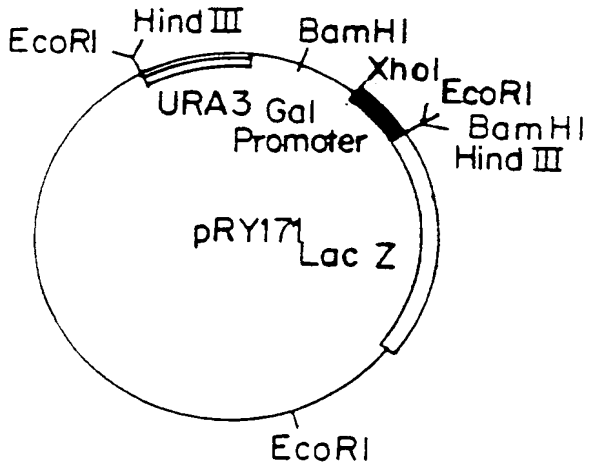

FIG. 2

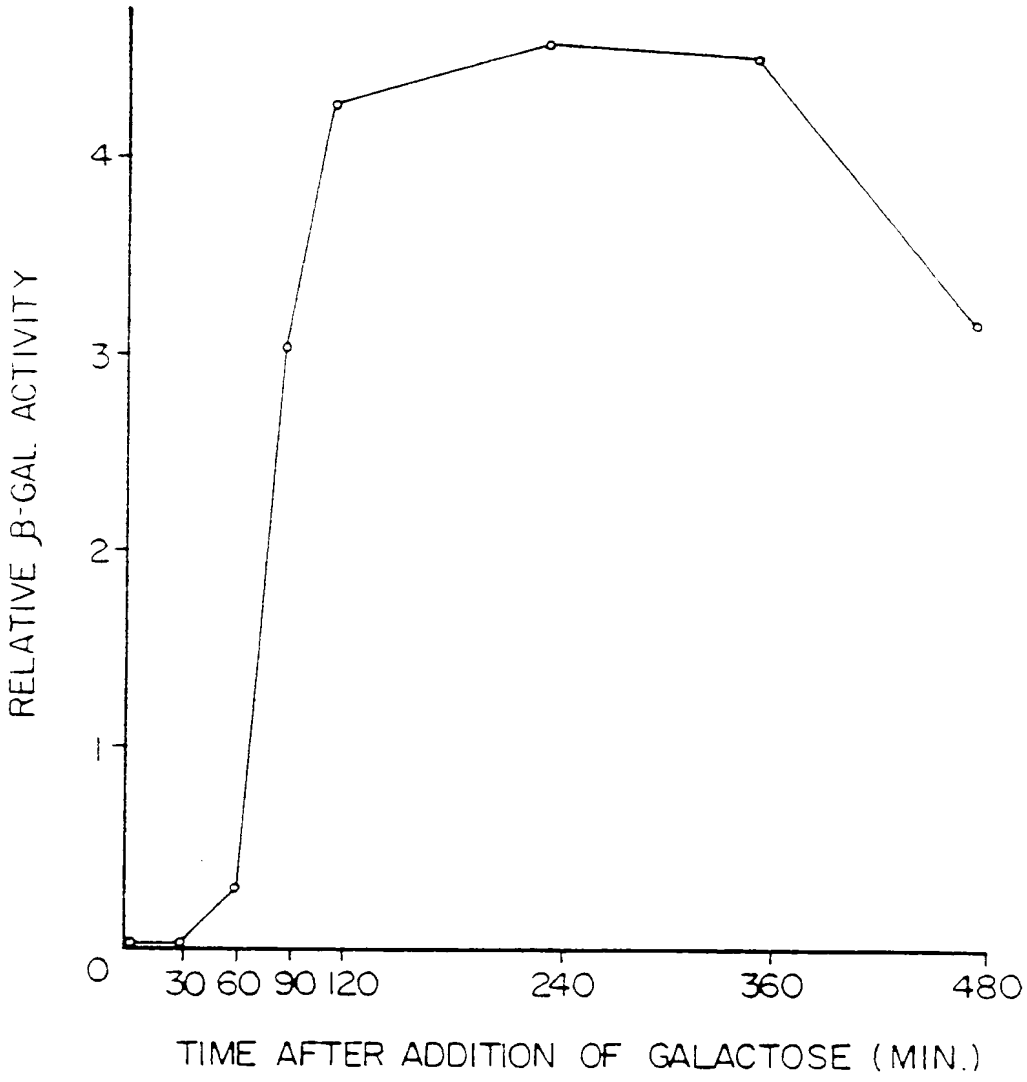

FIG. 3

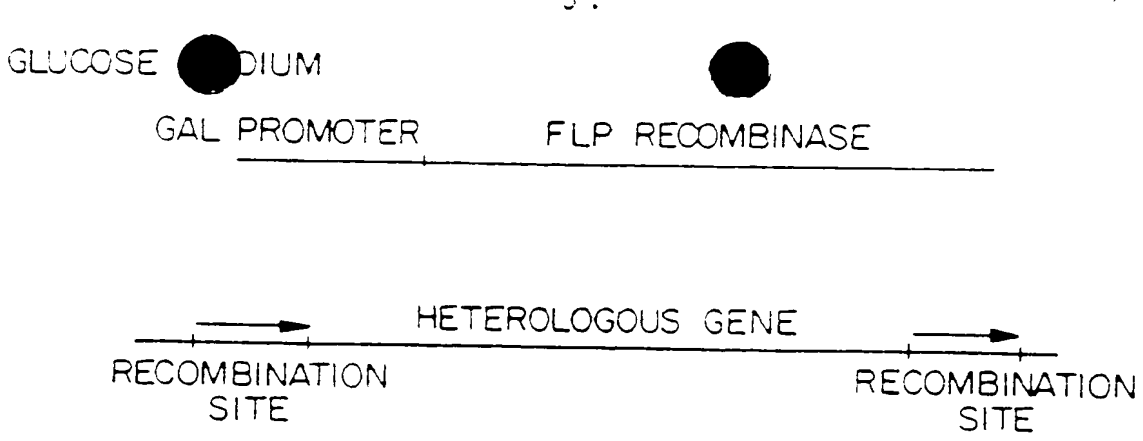

FIG. 4A

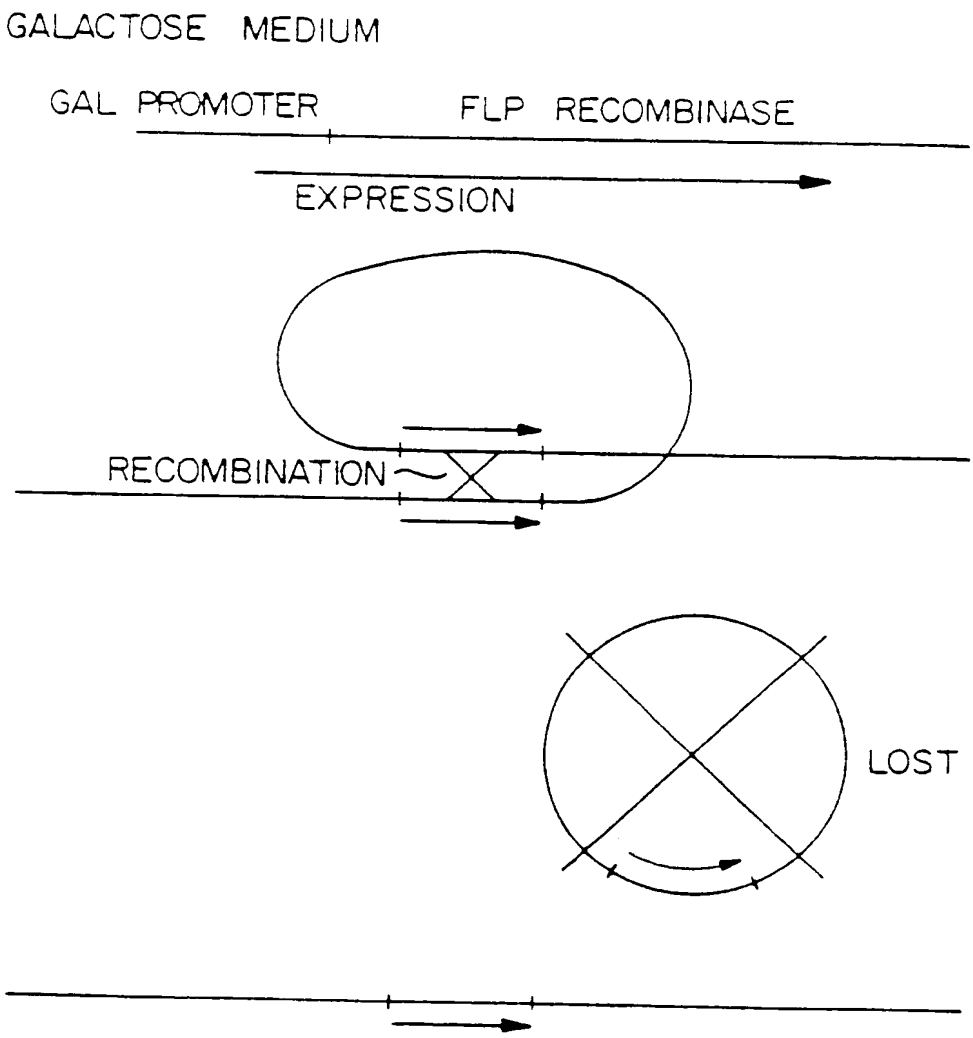

FIG. 4B

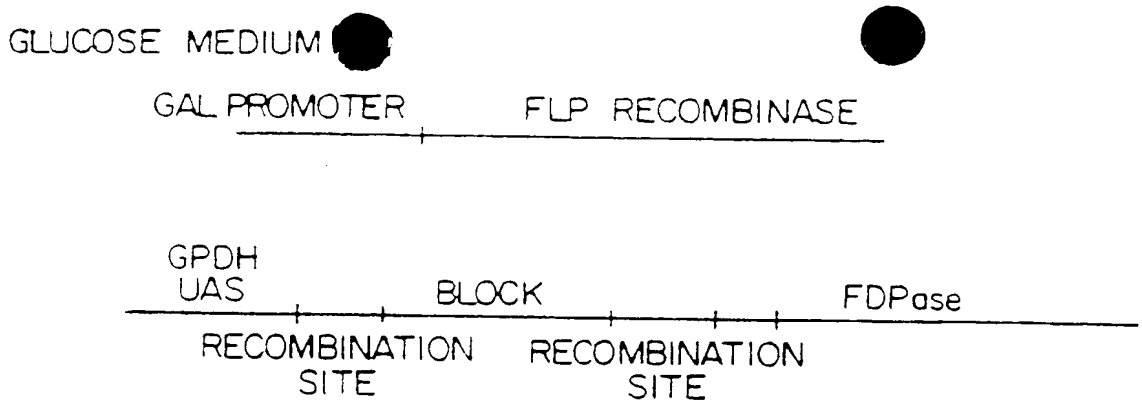

FIG. 5A

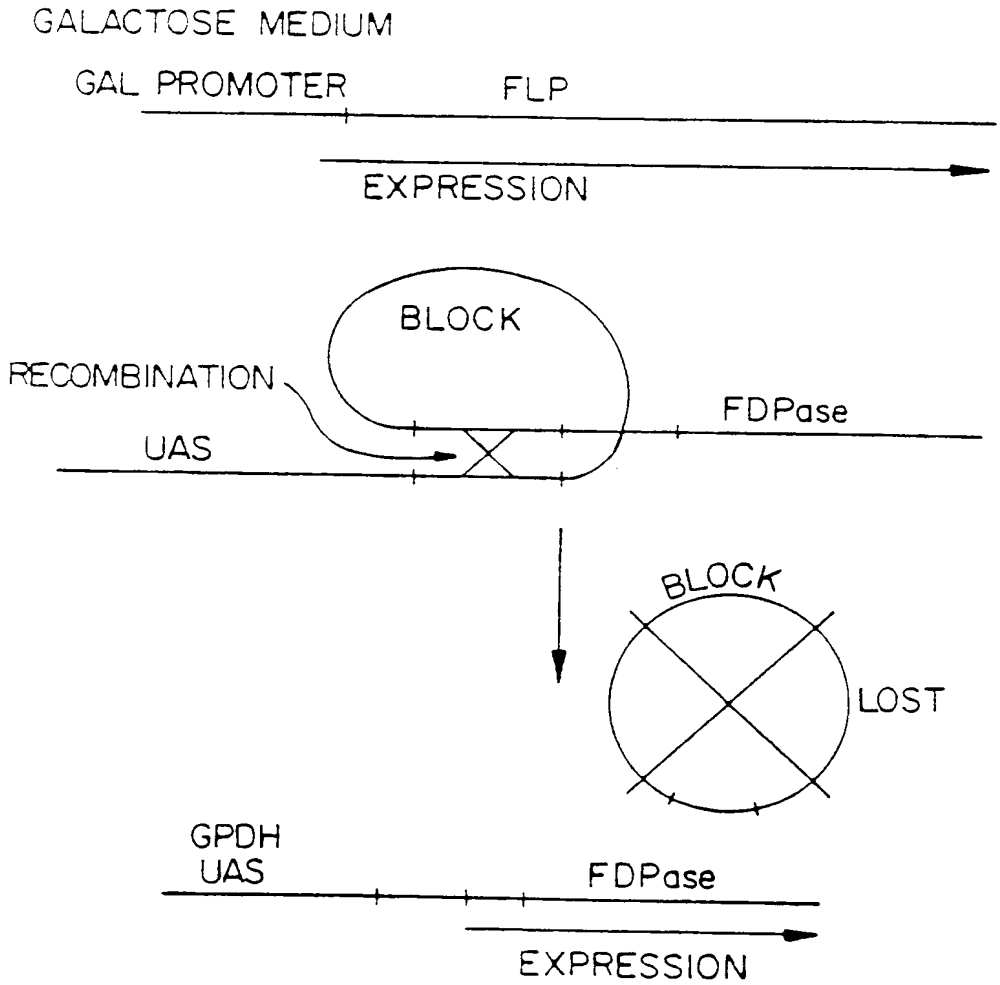

FIG. 5B

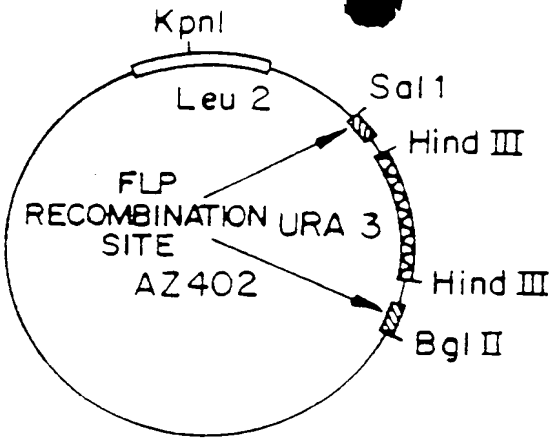

FIG. 6

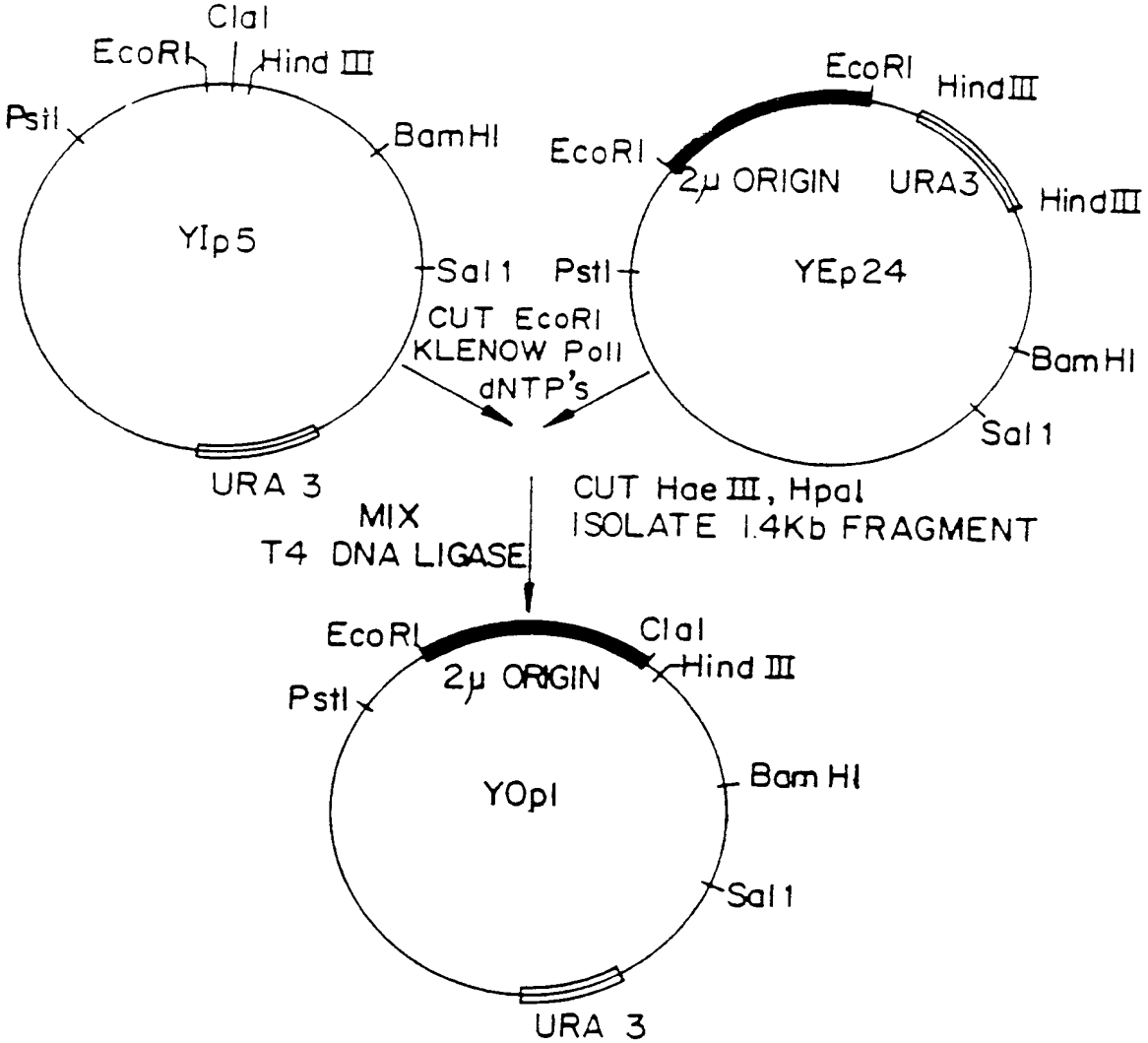

FIG. 7

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        10          20          30          40          50          60          70
ACCTGCTTAA GCAAATGCGC TTAAAAGCCG AACGCTCTAC CAACTGAGCT AACAAGGATG AGTTCTTCGA

        80          90         100         110         120         130         140
ATTTTCCAGT CTAAGATAGA CAACCCATCA AACTGCATGG TCCCGGGCTA ACTTCTGCTC TCTTTTCCGG

       150         160         170         180         190         200         210
ACGGATGGAA TCGCCGCTTT TGAATTCACC TCCGGGGTAT TATTATTATT CTTAGTAGTC GCGGTCGTGC

       220         230         240         250         260         270         280
GGACACCCGG AGTTATGCGG GCCCGAAAGC TCATTATGTA GTAAAGCTAG GTAATGTTAA GGGCGTAAGA

       290         300         310         320         330         340         350
GCCAACGCAA GGCAGCAATA GCCTGGTATT CCCACATATC AAGAAAGCTT AAAAAGTTGA GACAGGGAAT

       360         370         380         390         400         410         420
TTGAAGGCGA AGATTGCCGA ACTGGCCAAT ACCCACTACT TTTTTTTTGG TTTGCTTGGT TTATTCCTGT

       430         440         450         460         470         480         490
CGCTTGCCAA CTTGTGGCAT CTTCCCCACA CTATATTATA AGGATCGTCC TATGTATAGG CAATATTATC

       500         510         520         530         540         550         560
CATTTCACTC GCTAACAAAT GTACGTATAT ATATGGAGCA ACAAGTAGTG CAATTACAGA CGTGTATTTT

       570         580         590         600         610         620         630
GTCTTGATCT TGCTTTTTGT ATGATAGGCC TAAGAATAAC AGTGCGAACA TATAAGAAAC ATCCCTCATA

       640                     656                     671                     686
CTACCACACA T ATG CCA ACT CTA GTA AAT GGA CCA AGA AGA GAC TCT ACC GAA GGG
             MET Pro Thr Leu Val Asn Gly Pro Arg Arg Asp Ser Thr Glu Gly

                   701                     716                     731
TTT GAT ACC GAT ATC ATC ACT CTT CCT AGA TTC ATA ATC GAG CAC CAG AAG CAA
Phe Asp Thr Asp Ile Ile Thr Leu Pro Arg Phe Ile Ile Glu His Gln Lys Gln

    746                     761                     776                     791
TTT AAG AAC GCT ACT GGT GAT TTC ACA TTA GTA CTG AAT GCC TTG CAA TTC GCG
Phe Lys Asn Ala Thr Gly Asp Phe Thr Leu Val Leu Asn Ala Leu Gln Phe Ala

              806                     821                     836
TTC AAA TTT GTA TCT CAC ACC ATC AGA CGT GCT GAA TTG GTT AAC TTG GTT GGG
Phe Lys Phe Val Ser His Thr Ile Arg Arg Ala Glu Leu Val Asn Leu Val Gly
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Fig. 8A

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851                 866                 881                 896
TTA GCA GGC GCT TCC AAC TTC ACT GGT GAC CAG CAA AAG AAG TTG GAC GTT CTA
Leu Ala Gly Ala Ser Asn Phe Thr Gly Asp Gln Gln Lys Lys Leu Asp Val Leu

        911                 926                 941                 956
GGT GAT GAA ATA TTT ATC AAT GCC ATG AGG GCT AGT GGG ATC ATC AAG GTC CTT
Gly Asp Glu Ile Phe Ile Asn Ala MET Arg Ala Ser Gly Ile Ile Lys Val Leu

                971                 986                1001
GTA TCT GAA GAA CAG GAA GAC TTG ATC GTT TTT CCC ACA AAC ACG GGC TCA TAC
Val Ser Glu Glu Gln Glu Asp Leu Ile Val Phe Pro Thr Asn Thr Gly Ser Tyr

   1016                1031                1046                1061
GCA GTG TGT TGT GAT CCT ATT GAT GGC TCC TCA AAT TTG GAC GCC GGT GTC TCC
Ala Val Cys Cys Asp Pro Ile Asp Gly Ser Ser Asn Leu Asp Ala Gly Val Ser

           1076                1091                1106
GTT GGA ACT ATC GCG TCT ATA TTC AGA CTG CTA CCA GAC TCA TCA GGT ACT ATA
Val Gly Thr Ile Ala Ser Ile Phe Arg Leu Leu Pro Asp Ser Ser Gly Thr Ile

1121                1136                1151                1166
AAC GAC GTA CTG AGA TGT GGT AAA GAA ATG GTA GCC GCT TGC TAT GCC ATG TAC
Asn Asp Val Leu Arg Cys Gly Lys Glu MET Val Ala Ala Cys Tyr Ala MET Tyr

        1181                1196                1211                1226
GGA TCC TCT ACG CAT CTA GTA TTG ACA TTG GGT GAT GGA GTT GAT GGG TTT ACC
Gly Ser Ser Thr His Leu Val Leu Thr Leu Gly Asp Gly Val Asp Gly Phe Thr

                1241                1256                1271
TTA GAC ACA AAC TTG GGC GAA TTC ATC TTG ACT CAT CCT AAC TTA AGA ATT CCG
Leu Asp Thr Asn Leu Gly Glu Phe Ile Leu Thr His Pro Asn Leu Arg Ile Pro

   1286                1301                1316                1331
CCT CAA AAG GCC ATC TAC TCA ATT AAT GAA GGT AAC ACC CTC TAC TGG AAC GAG
Pro Gln Lys Ala Ile Tyr Ser Ile Asn Glu Gly Asn Thr Leu Tyr Trp Asn Glu

           1346                1361                1376
ACT ATA AGA ACA TTT ATT GAG AAA GTC AAA CAA CCC CAA GCA GAC AAC AAC AAC
Thr Ile Arg Thr Phe Ile Glu Lys Val Lys Gln Pro Gln Ala Asp Asn Asn Asn

1391                1406                1421                1436
AAG CCT TTC TCG GCT AGG TAT GTT GGA TCC ATG GTT GCT GAT GTT CAC AGG ACG
Lys Pro Phe Ser Ala Arg Tyr Val Gly Ser MET Val Ala Asp Val His Arg Thr

        1451                1466                1481                1496
TTT CTT TAC GGT GGC CTT TTC GCA TAC CCT TGC GAC AAG AAG AGC CCC AAC GGA
Phe Leu Tyr Gly Gly Leu Phe Ala Tyr Pro Cys Asp Lys Lys Ser Pro Asn Gly

                1511                1526                1541
AAA CTG AGG TTG CTT TAT GAG GCC TTC CCA ATG GCT TTC TTA ATG GAA CAA GCA
Lys Leu Arg Leu Leu Tyr Glu Ala Phe Pro MET Ala Phe Leu MET Glu Gln Ala
```

Fig. 8B

```
   1556                1571                1586                1601
GGG GGA AAA GCG GTC AAC GAT CGC GGA GAG AGA ATC TTG GAT TTG GTG CCA AGT
Gly Gly Lys Ala Val Asn Asp Arg Gly Glu Arg Ile Leu Asp Leu Val Pro Ser

           1616                1631                1646
CAT ATC CAT GAC AAA TCT TCT ATT TGG TTG GGT TCT TCA GGT GAA ATT GAC AAA
His Ile His Asp Lys Ser Ser Ile Trp Leu Gly Ser Ser Gly Glu Ile Asp Lys

1661                1676                1695       1705       1715       1725
TTT TTA GAC CAT ATT GGC AAG TCA CAG TAGTTCAATG ATCGCCTTCT TTTCTTATTT TCTTTGTTCT
Phe Leu Asp His Ile Gly Lys Ser Gln

      1735       1745       1755       1765       1775       1785       1795
GTACTTTAGT ACGCGAAAAA AAAAAATCTG TATATGTCCT TATATATATA TATATTTATA TATATATATG

      1805       1815       1825       1835       1845       1855       1865
TGTATGTATG TGTACCGTAA GCATTACTCC TTCTAATAAT GAAAATTCTT AGGAAAAGAG AAAGGAAGTA

      1875       1885       1895       1905       1915       1925       1935
GCGAATGGAA TGGGATGGAA GTTTTAAAGA ACATTAGAAT TTATCCTTTG TCAAACTTCA TCACATCAAC

      1945       1955       1965       1975       1985       1995       2005
CAAGAACTAT ATAAACCTAC CAAATGAATT AAGAAACCTA ATTAGTGAAG AGCAGGAGAG TAAACTAGGG

      2015       2025       2035       2045       2055       2065       2075
TTCTTGCACA TCATTGAAAG TGATTTTAAA CCTTCGGTAG CGCTGCAAAA GTTGGTGAAT TGTACTACGG

      2085       2095       2105       2115       2125       2135       2145
GGGACGAAAA GATCCTAATC ATAGATATAG TATCAATATG GTCCCAACAA AAGCAAAGAC AGCATGGCGC

      2155       2165       2175       2185       2195
GATCTACATG AATTCGCTAT CTTGCATAAA CATCACGGGA TTAATCGTAT TTCTAGA
```

Fig. 8C

Fig. 9A

yeast fdp

```
                10                                                  20
Met Pro Thr Leu Val Asn Gly Pro Arg Arg Asp Ser Thr Glu Gly Phe Asp Thr Asp Ile

                30                                                  40
Ile Thr Leu Pro Arg Phe Ile Ile Glu His Gln Lys Gln Phe Lys Asn Ala Thr Gly Asp

                50                                                  60
Phe Thr Leu Val Leu Leu Asn Ala Leu Gln Phe Ala Phe Lys Phe Val Ser His Thr Ile

                70                                                  80
Arg Arg Ala Glu Leu Val Asn Leu Val Gly Leu Ala Gly Ala Ser Asn Phe Thr Gly Asp

                90                                                  100
Gln Gln Lys Lys Leu Asp Val Leu Gly Asp Glu Ile Phe Ile Asn Ala Met Arg Ala Ser

                110                                                 120
Gly Ile Ile Lys Val Leu Val Ser Glu Glu Gln Glu Asp Leu Ile Val Phe Pro Thr Asn

                130                                                 140
Thr Gln Ser Tyr Ala Val Cys Cys Asp Pro Ile Asp Gly Ser Ser Asn Leu Asp Ala Gly

                150                                                 160
Val Ser Val Gly Thr Ile Ala Ser Ile Phe Arg Leu Leu Pro Asp Ser Ser Gly Thr Ile

                170                                                 180
Asn Asp Val Leu Arg Cys Gly Lys Glu Met Val Ala Ala Cys Tyr Ala Met Tyr Gly Ser

                190                                                 200
Ser Thr His Leu Val Leu Thr Leu Gly Asp Gly Val Asp Gly Phe Thr Leu Asp Thr Asn

                210                                                 220
Leu Gly Glu Phe Ile Leu Thr His Pro Asn Leu Arg Ile Pro Pro Gln Lys Ala Ile Tyr

                230                                                 240
Ser Ile Asn Glu Gly Asn Thr Leu Tyr Trp Asn Glu Thr Ile Arg Thr Phe Ile Glu Lys
```

Fig. 9A (cont'd.)

```
                                                    250                                                 260
Val Lys Gln Pro Gln Ala Asp Asn Asn Asn Lys Pro Phe Ser Ala Arg Tyr Val Gly Ser

                                                    270                                                 280
Met Val Ala Asp Val His Arg Thr Phe Val Tyr Gly Gly Leu Phe Ala Tyr Pro Cys Asp

                                                    290                                                 300
Lys Lys Ser Pro Asn Gly Lys Leu Arg Leu Leu Tyr Glu Ala Phe Pro Met Ala Phe Leu

                                                    310                                                 320
Met Glu Gln Ala Gly Gly Lys Ala Val Asn Asp Arg Gly Glu Arg Ile Leu Asp Leu Val

                                                    330                                                 340
Pro Ser His Ile His Asp Lys Ser Ser Ile Trp Leu Gly Ser Ser Gly Glu Ile Asp Lys

Phe Leu Asp His Ile Gly Lys Ser Gln
```

Fig. 9B

pig fdp

```
                                                  10                  20
Thr Asp Gln Ala Ala Phe Asp Thr Asn Ile Val Thr Leu Thr Arg Phe Val Met Glu Gln

                                                  30                  40
Gly Arg Lys Ala Arg Gly Thr Gly Glu Met Thr Gln Leu Leu Asn Ser Leu Cys Thr Ala

                                                  50                  60
Val Lys Ala Ile Ser Thr Ala Val Arg Lys Ala Gly Ile Ala His Leu Tyr Gly Ile Ala

                                                  70                  80
Gly Ser Thr Asn Val Thr Gly Asp Gln Val Lys Lys Leu Asp Val Leu Ser Asn Asp Leu

                                                  90                 100
Val Ile Asn Val Leu Lys Ser Ser Phe Ala Thr Cys Val Leu Val Thr Glu Glu Asp Lys

                                                 110                 120
Asn Ala Ile Ile Val Glu Pro Glu Lys Arg Gly Lys Tyr Val Val Cys Phe Asp Pro Leu

                                                 130                 140
Asp Gly Ser Ser Asn Ile Asp Cys Leu Val Ser Ile Gly Thr Ile Phe Gly Ile Tyr Arg

                                                 150                 160
Lys Asn Ser Thr Asp Glu Pro Ser Glu Lys Asp Ala Leu Gln Pro Glu Arg Asn Leu Val

                                                 170                 180
Ala Ala Gly Tyr Ala Leu Tyr Gly Ser Ala Thr Met Leu Val Leu Ala Met Val Asn Gly

                                                 190                 200
Val Asn Cys Phe Met Leu Asp Pro Ala Ile Gly Glu Phe Ile Leu Val Asp Arg Asn Val

                                                 210                 220
Lys Ile Lys Lys Lys Gly Ser Ile Tyr Ser Ile Asn Glu Gly Tyr Ala Lys Glu Phe Asp
```

Fig. 9B (cont'd.)

```
                                                                  230                                                                  240
Pro Ala Ile Thr Glu Tyr Ile Glu Arg Lys Lys Phe Pro Pro Asp Asn Ser Ala Pro Tyr

                                                                  250                                                                  260
Gly Ala Arg Tyr Val Gly Ser Met Val Ala Asp Val His Arg Thr Leu Val Tyr Gly Gly

                                                                  270                                                                  280
Ile Phe Met Tyr Pro Ala Asn Lys Lys Ser Pro Lys Gly Lys Leu Arg Leu Leu Tyr Glu

                                                                  290                                                                  300
Cys Asn Pro Met Ala Tyr Val Met Glu Lys Ala Gly Gly Leu Ala Thr Thr Gly Lys Glu

                                                                  310                                                                  320
Ala Val Leu Asp Ile Val Pro Thr Asp Ile His Gln Arg Ala Pro Ile Ile Leu Gly Ser

                                                                  330
Pro Glu Asp Val Thr Glu Leu Leu Glu Ile Tyr Gln Lys His Ala
```

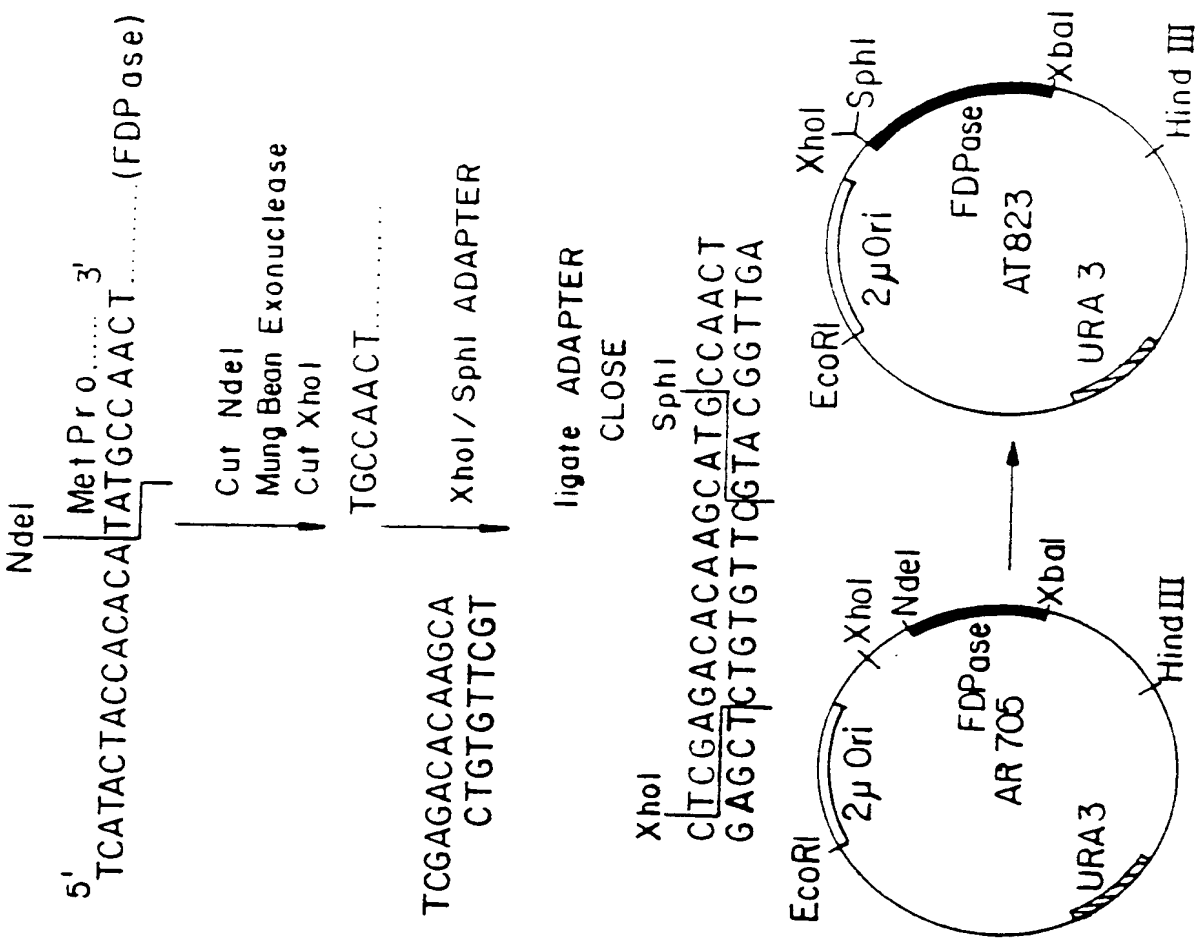

FIG. 10

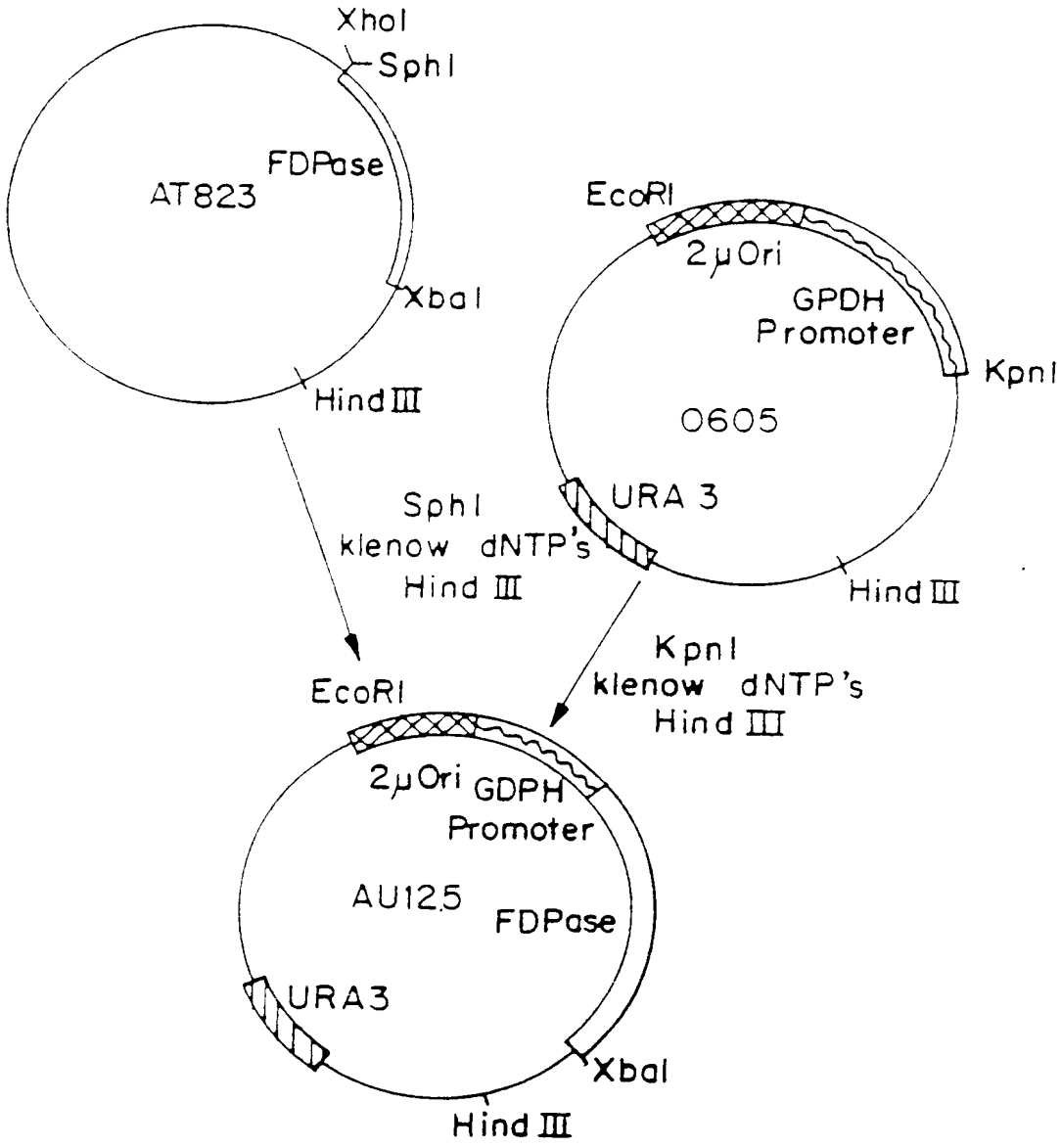

FIG. 11

EcoRV

GAT	ACC	GAT	ATC	ATC	ACT	CTT	DNA
Asp	Thr	Asp	Ile	Ile	Thr	Leu	AMINO ACIDS
17	18	19	20	21	22	23	RESIDUE

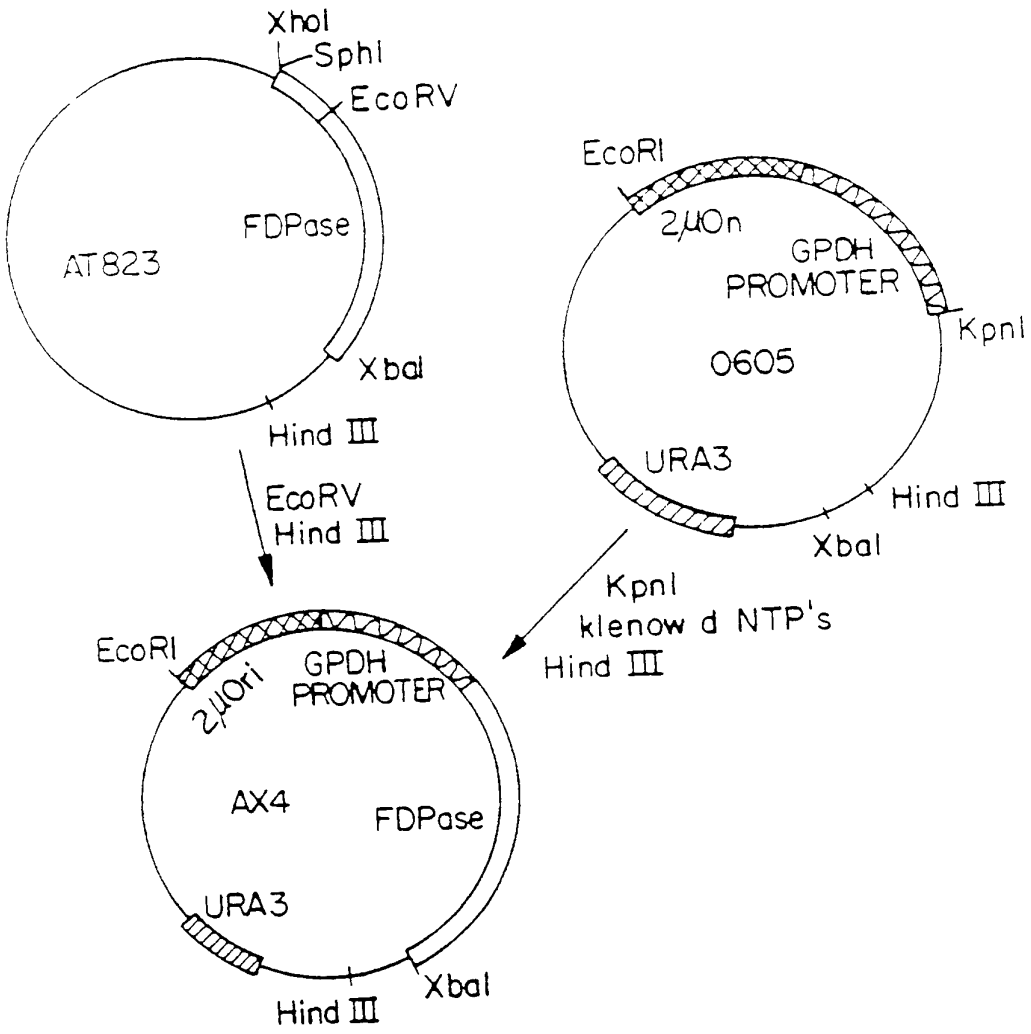

FIG. 12

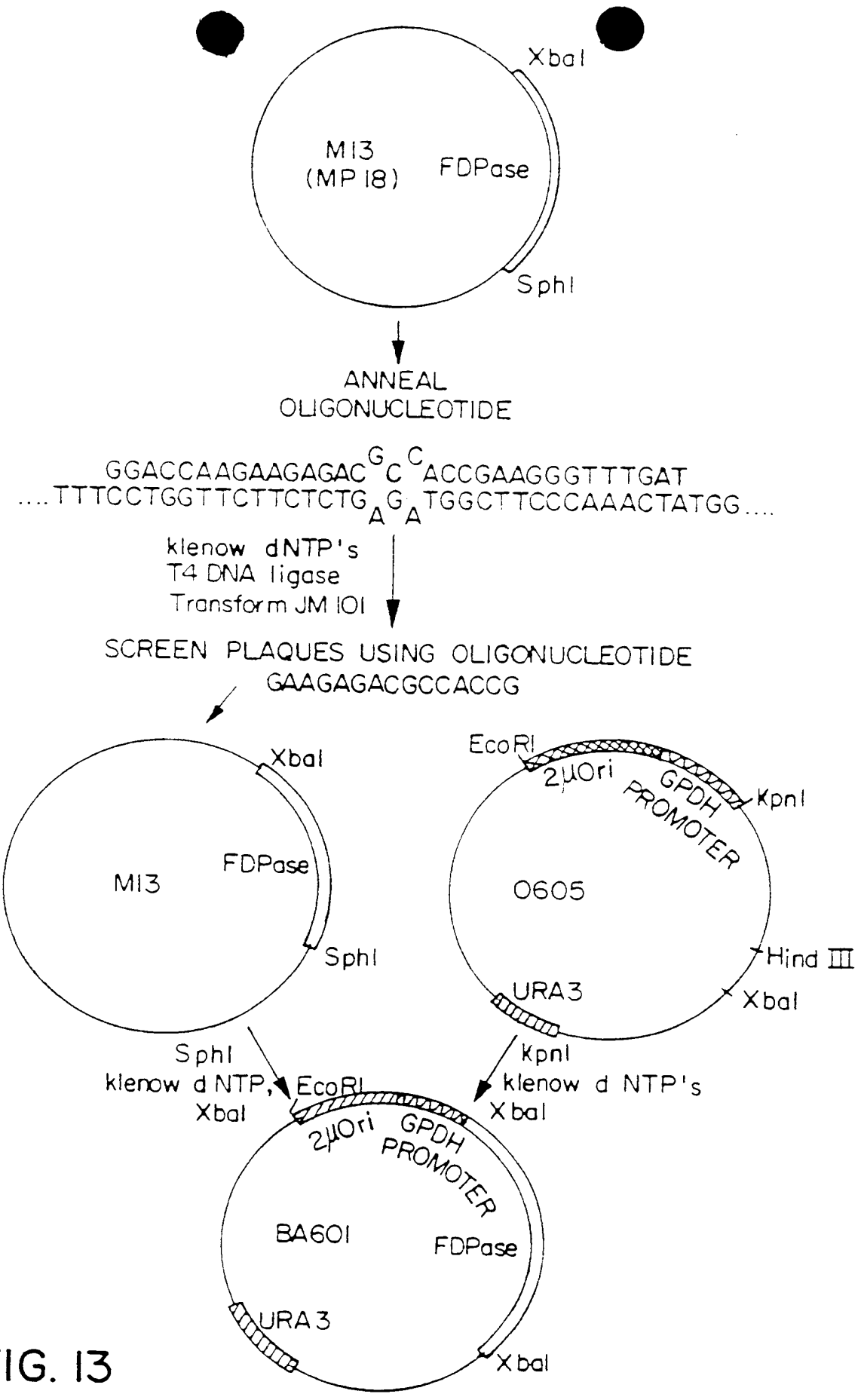

FIG. 13

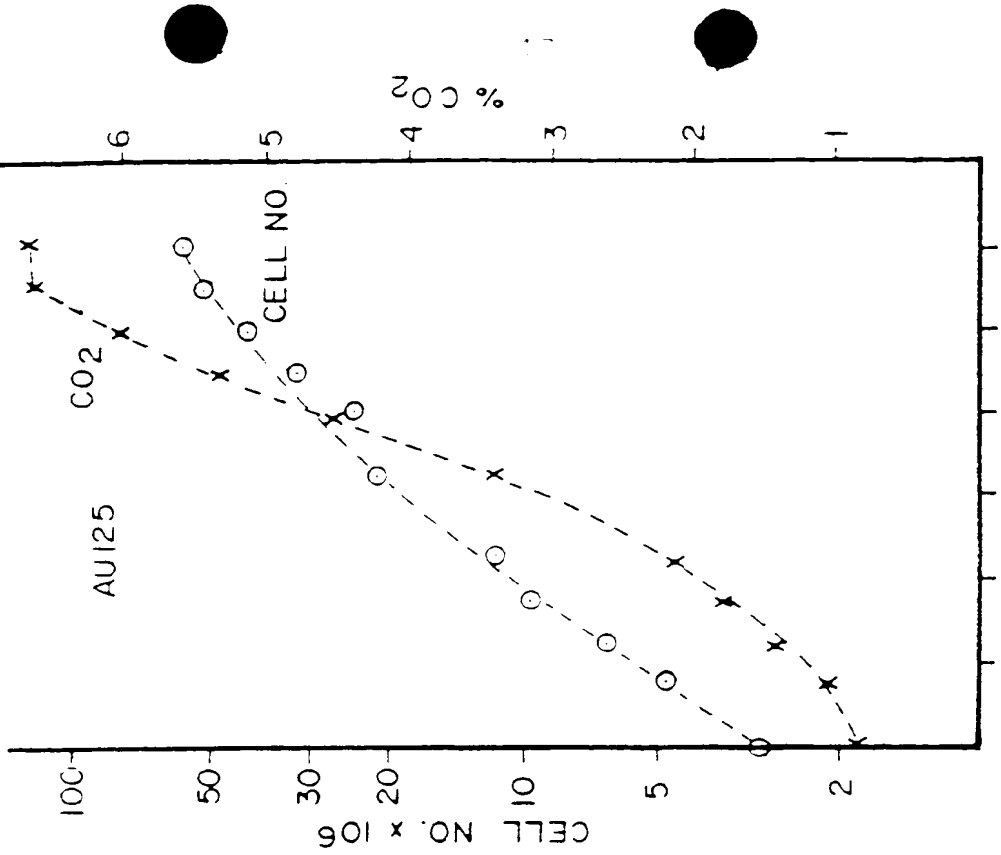

FIG. 14b

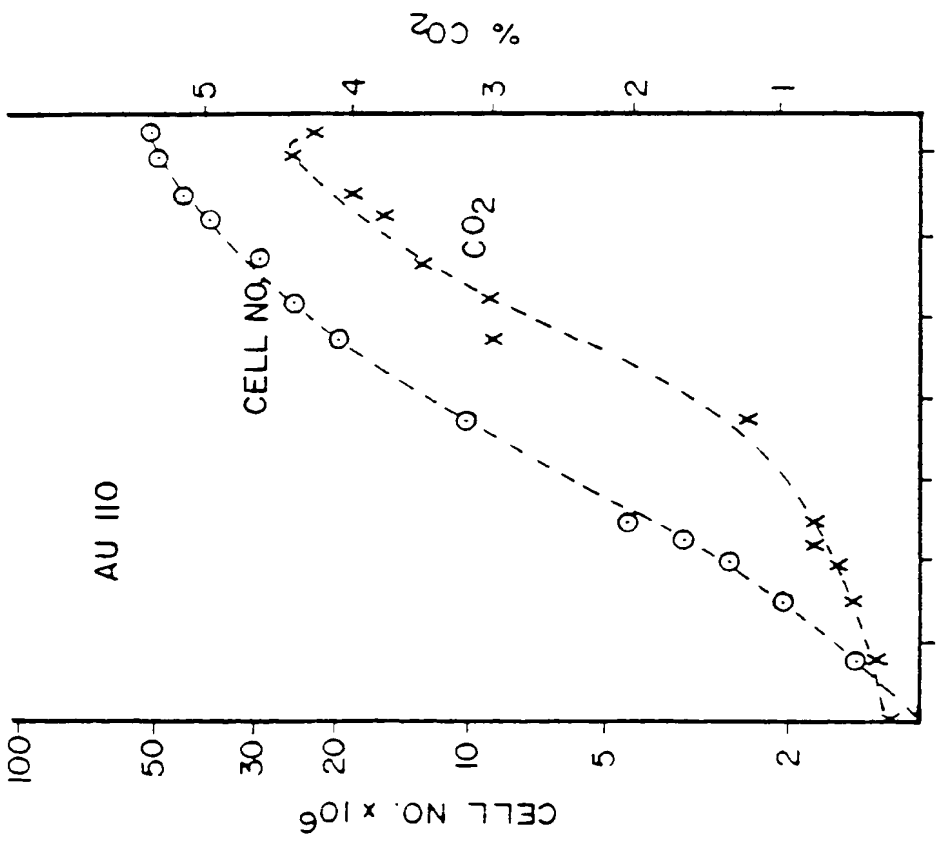

FIG. 14a

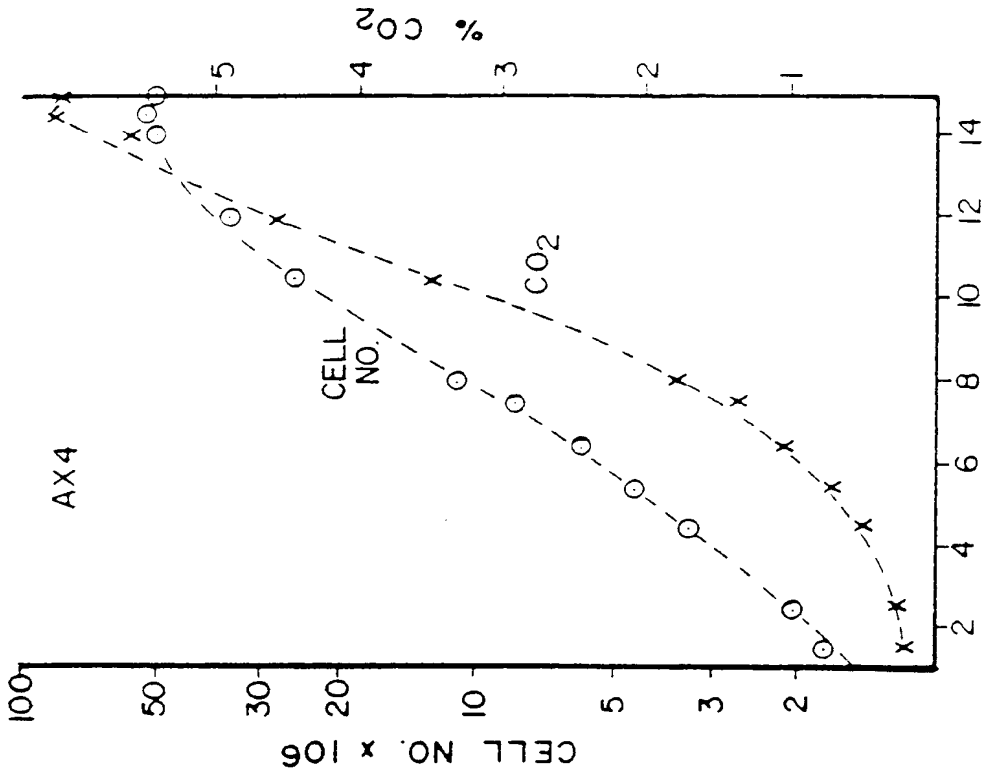

FIG. 14c

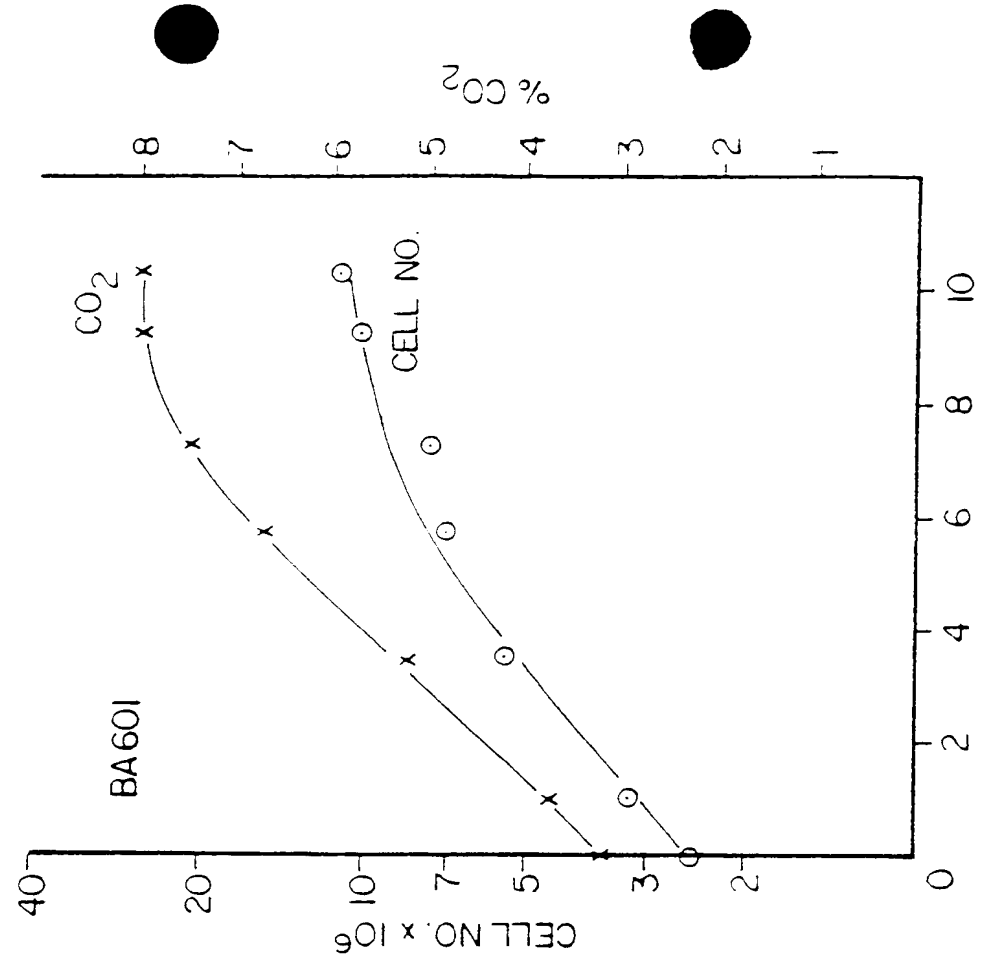

FIG. 15B

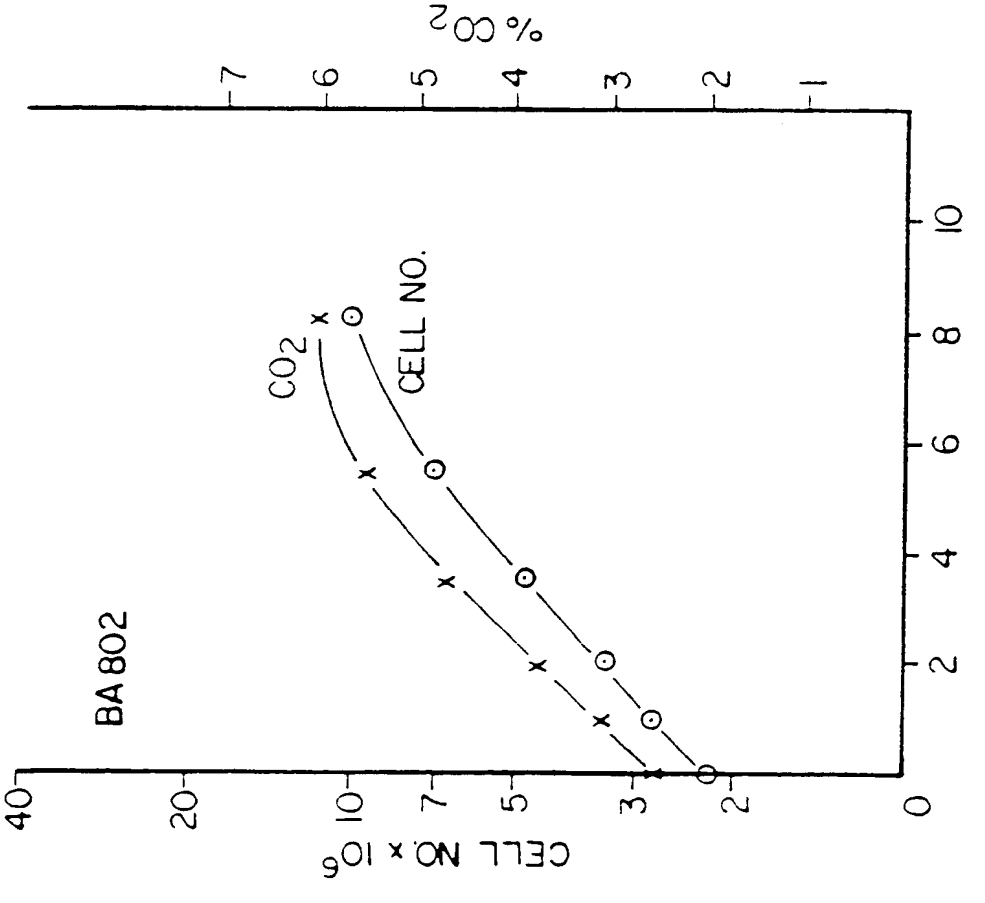

FIG. 15A

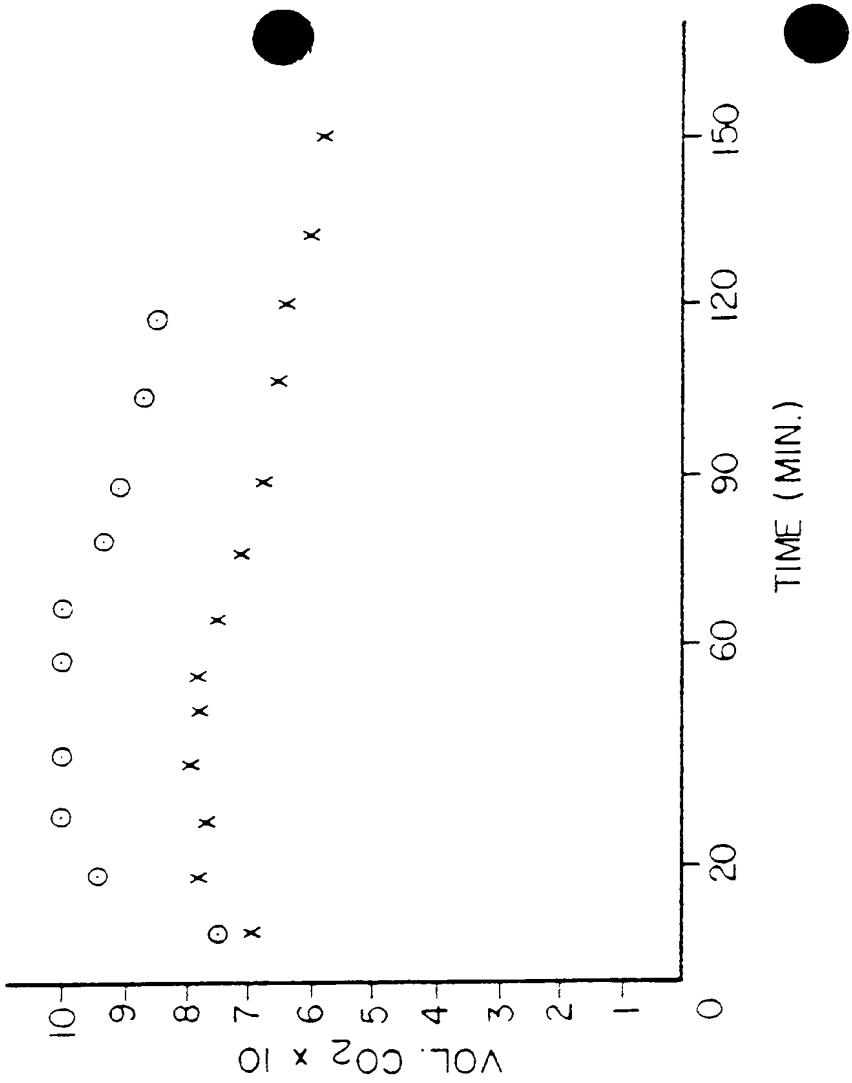

FIG. 17

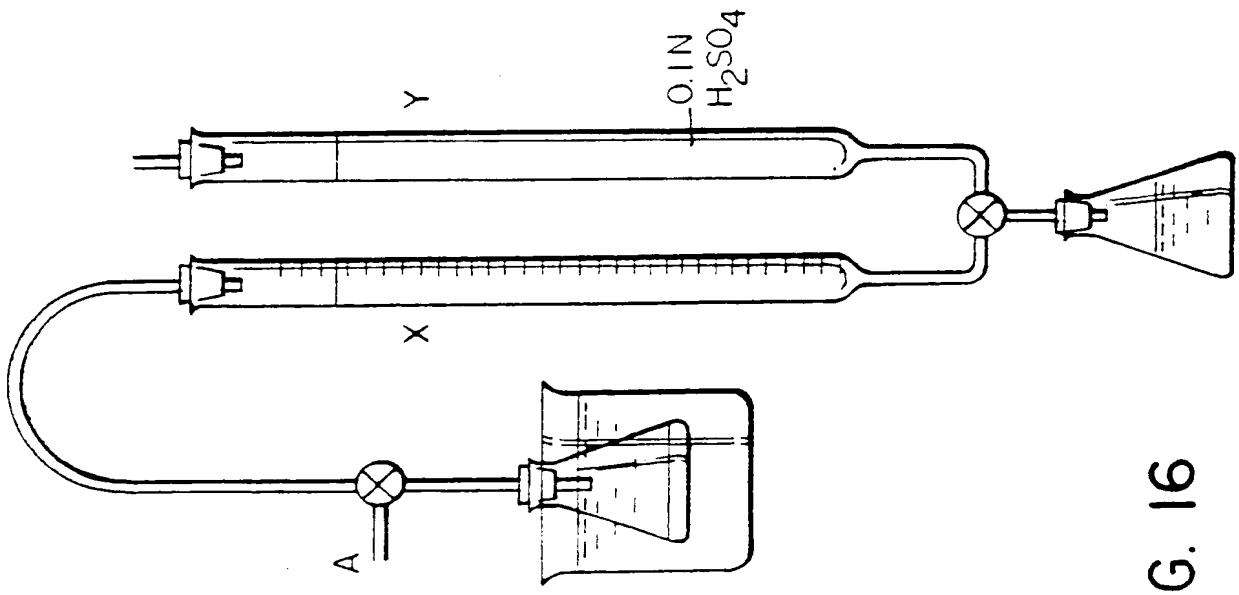

FIG. 16

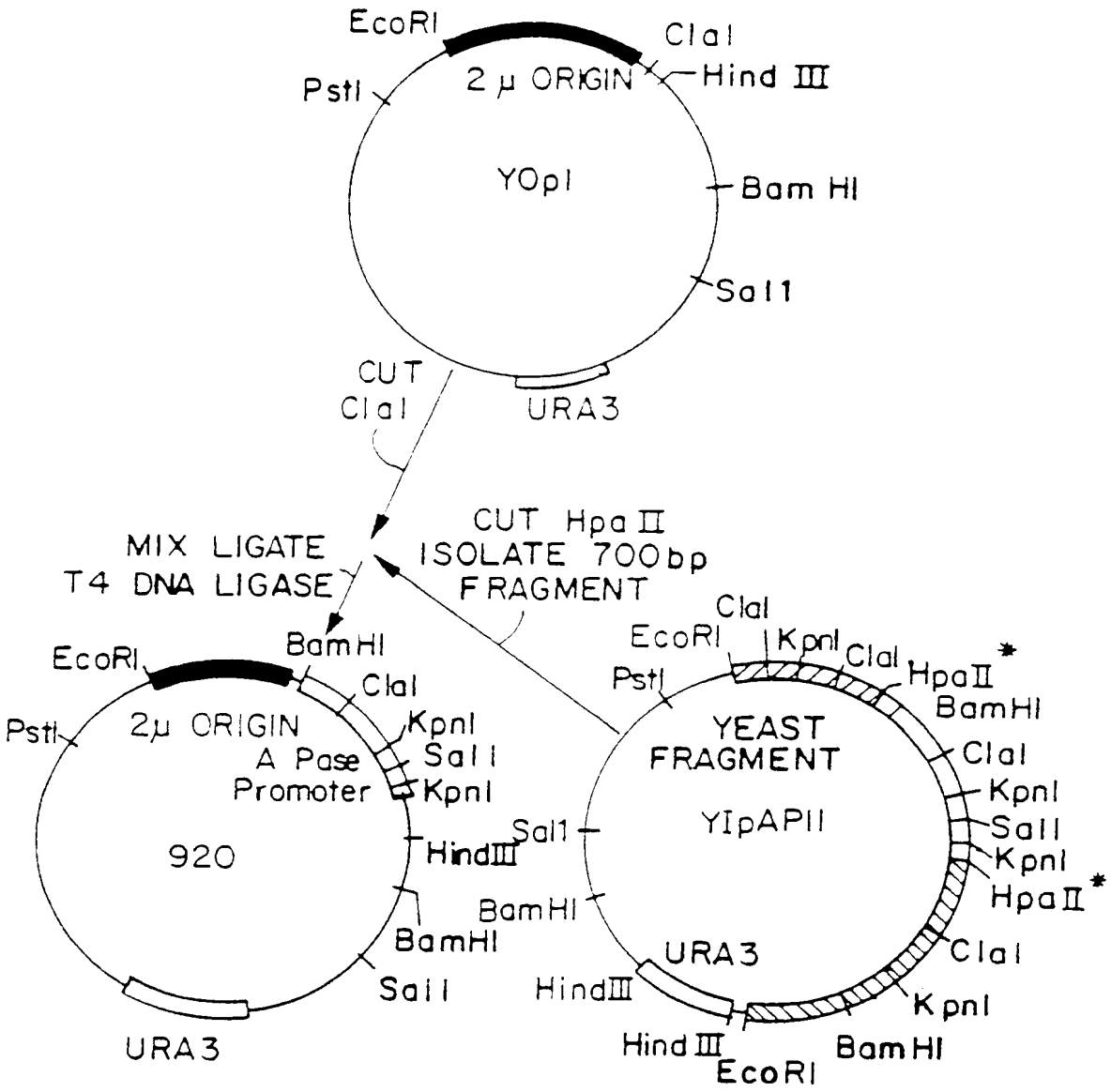

FIG. 18

Fig. 19

GGATCCGAAAGTTGTATTCAACAAGAATGCGCAAATATGTCAACGTATTTGGAAGTCAAC

TTATGTGCGCTGCTTTAATGTTTTCTCATGTAAGCGGACGTCGTCTATAAACTTCAA

ACGAAGCTAAAAGGTTCATAGCGCTTTTTTCTTTGTCTGCACAAAGAAATATATATTAAA

TTAGCACGTTTTCGCATAGAACGCAACGAACGCAACTGCACAATGCCAAAAAAAGTAAAA

CLA1

GTGATTAAAAGAGTTAATTGAATAGGCAATCTCTAAATGTATCGATACAACCTTGGCACT

CACACGTGGGACTAGCACAGACTAAATTTATGATTCTGGTCCCTGTTTTCGAAGAG

ATCGCACATGCCAAATTATCAAATTGGTCACCTTACTTGGCAAGGCATATACCCATTTGG

GAATAAAGGGTAAACACTTTGAATTGTCGAAATGAAACGTATATAAGCGCTGATGTTTTG

CTAAGTCGAGGTTAGTATGGCTTCATCTCTCATGAGAATAAGAACAACAAGAAATAGAGC

MetPheLysSerValValTyr.....

AAGCAAATTCGAGATTACCAATGTTTAAATCTGTTGTTTATTCAATTTTAGCCGCTTCT

Kpn1

TTGGCCAATGCAGGTACC

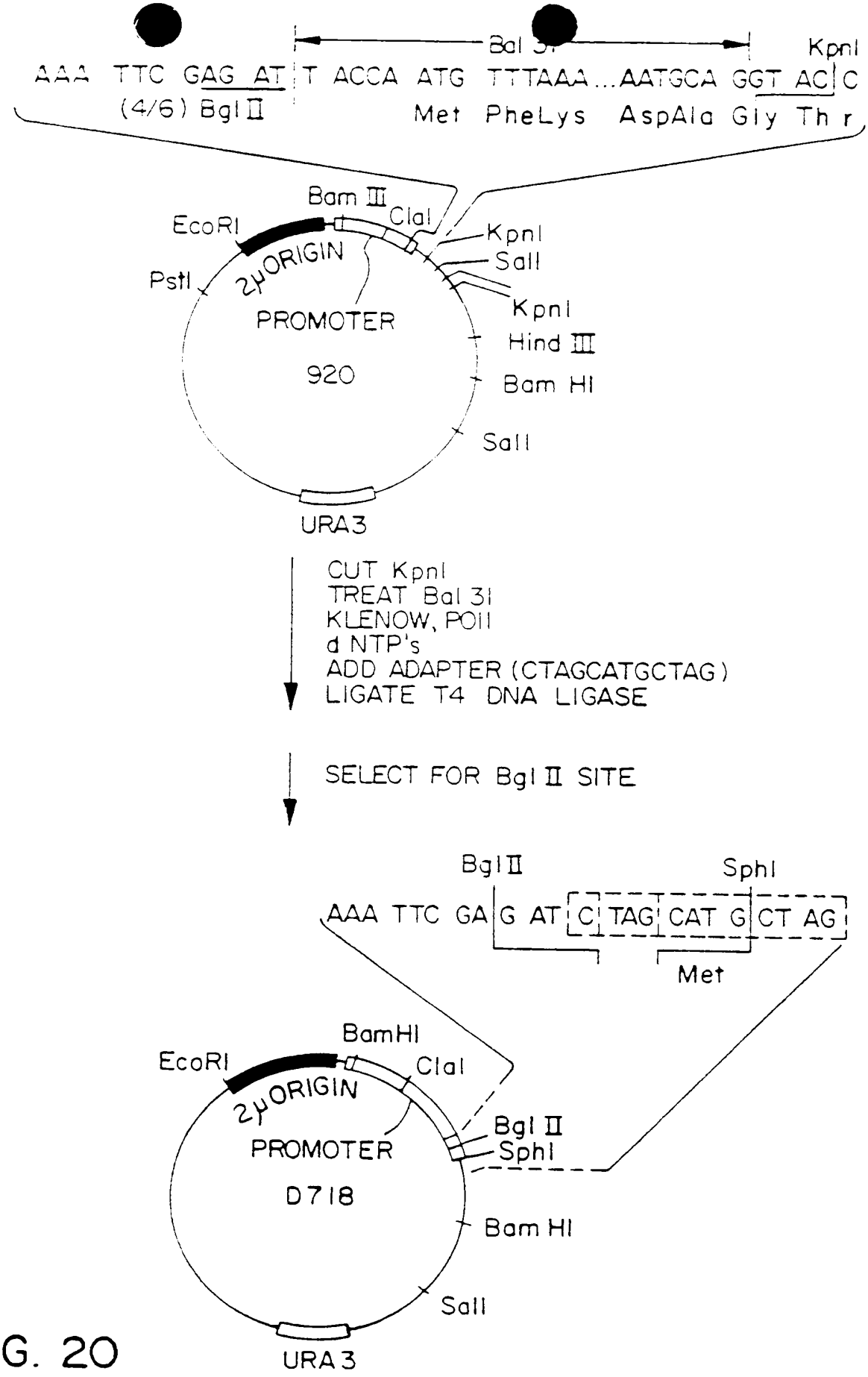

FIG. 20

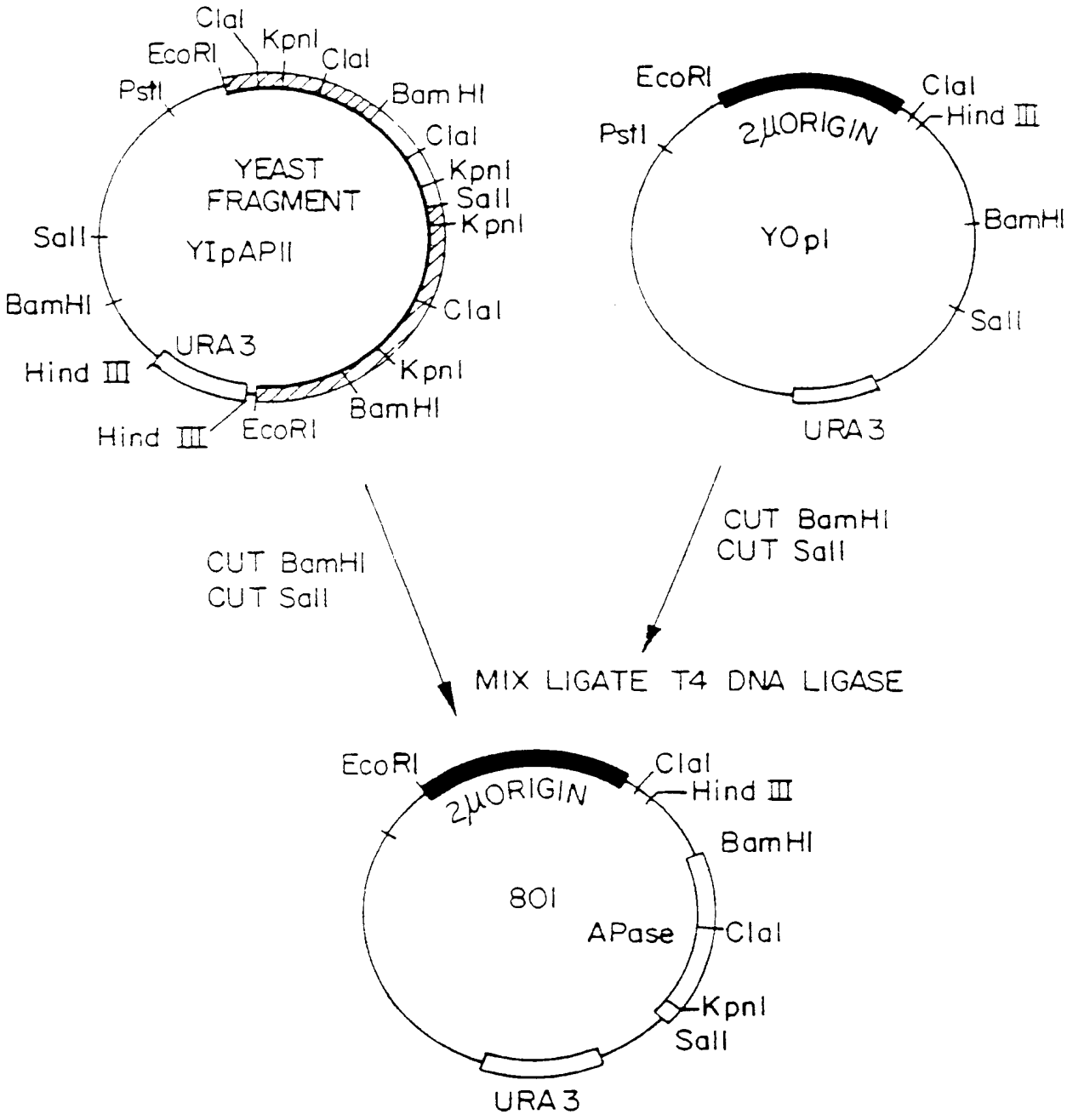

FIG. 21a

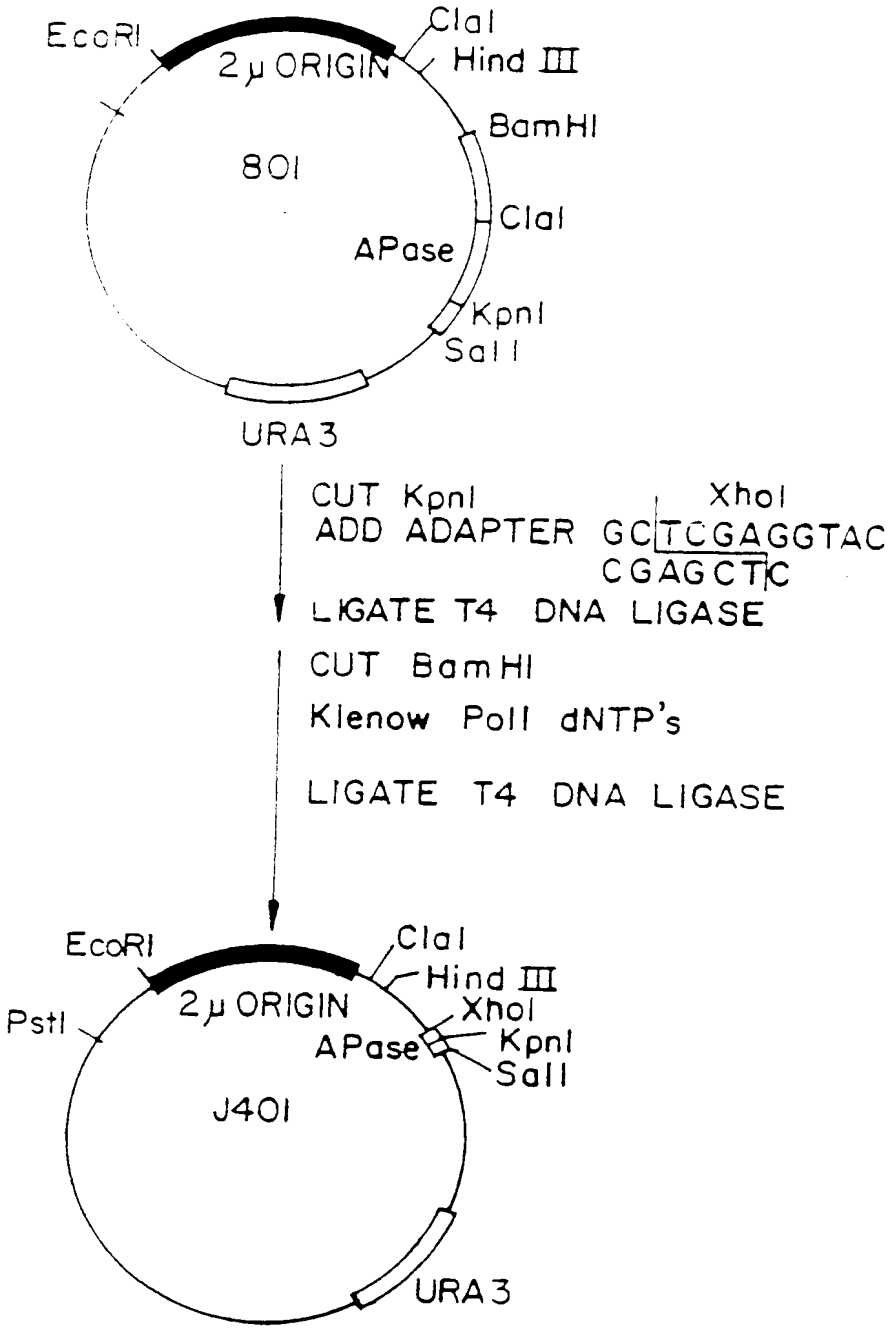

FIG. 21b

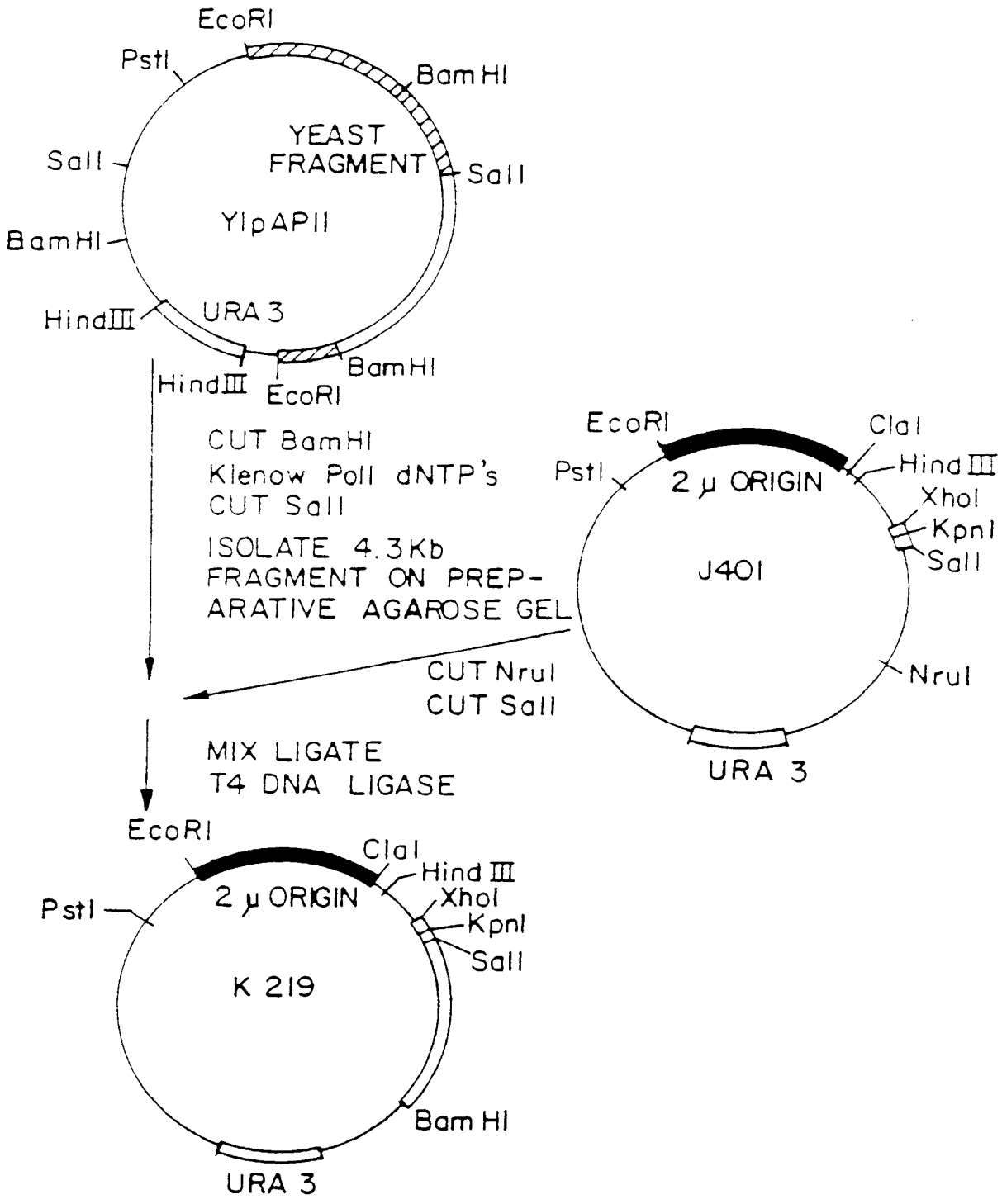

FIG. 22a

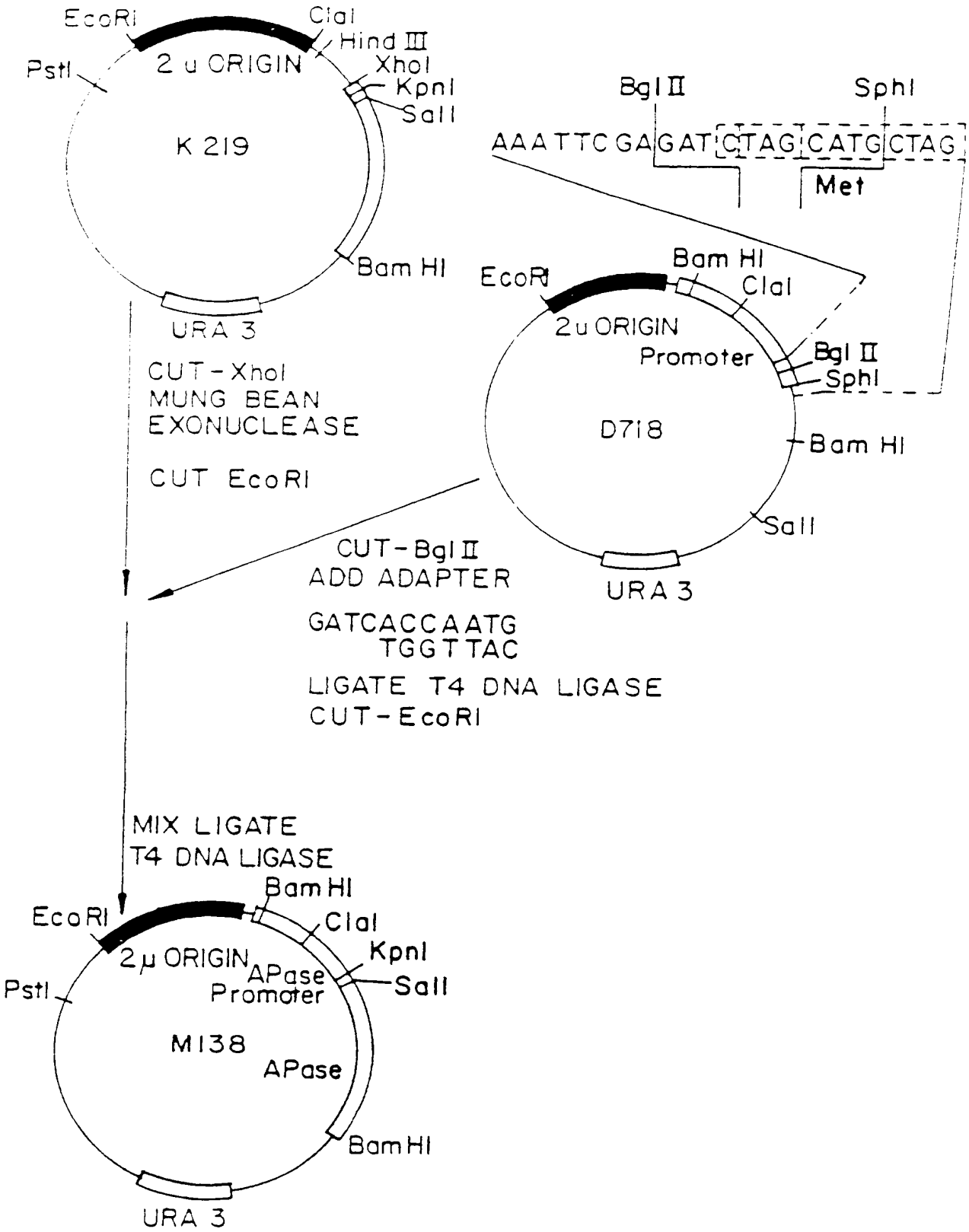

FIG. 22b

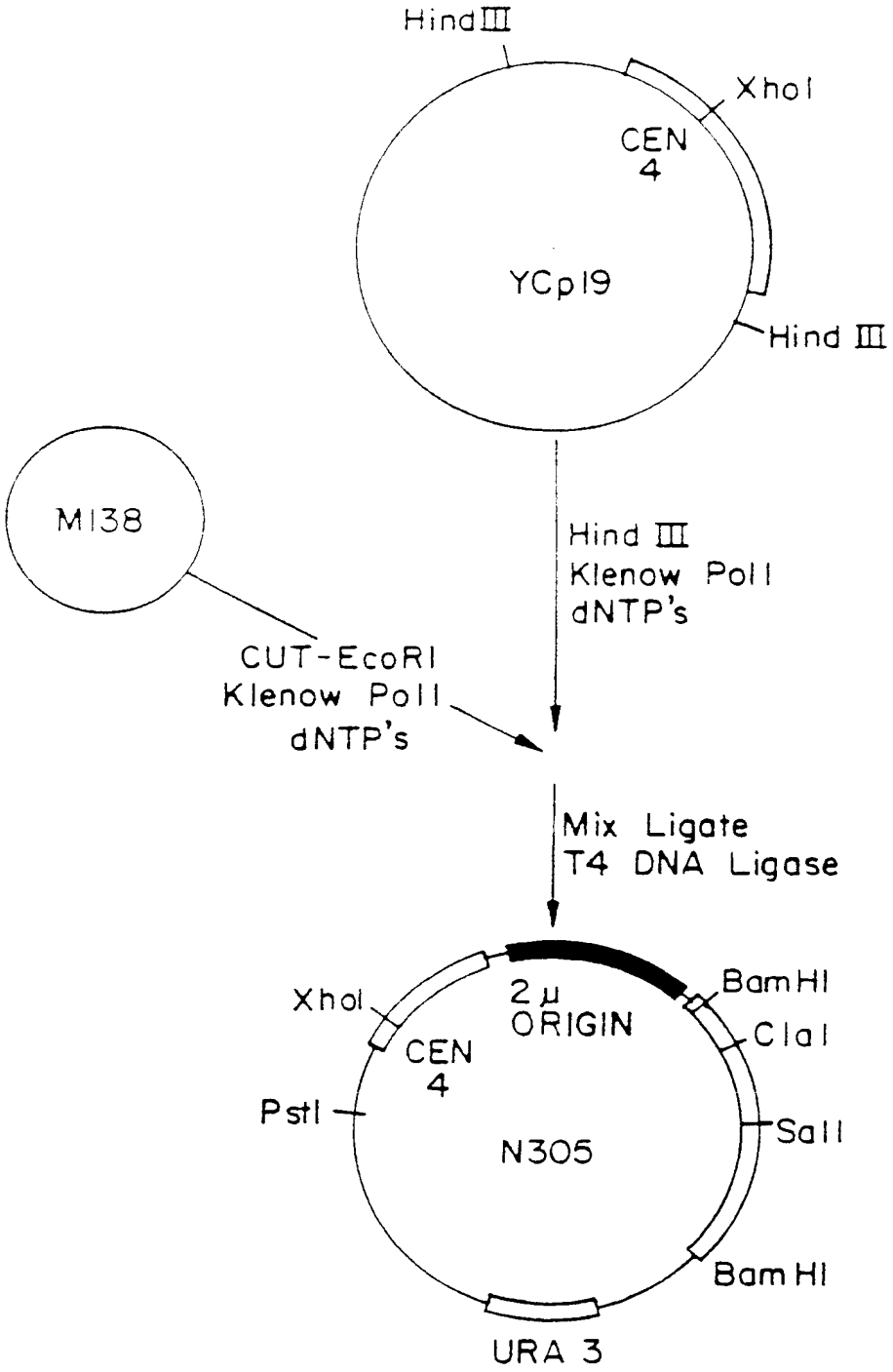

FIG. 23

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/02447

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12P 7/06; C12N 15/00, C12N 1/18, C12N 1/00
U.S. CL: 435/161, 172.3, 255, 317

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/161, 172.3, 255, 256, 244, 317 935/28, 37, 60, 69

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Chemical Abstracts Data Base (1967-1987), Biosis Data Base (1969-1987), Lexpat (1975-1987) Keywords: FLP,acid phosphatase, leader, signal, fructose 1,6-diphosphatase. See Attachment

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	A. LEHNINGER, Biochemistry, second edition. Published 1975 by Worth Publishers, Inc., New York, N.Y., USA. See pages 435, 438, 630, 631.	1,19
X A	Journal of Molecular Biology, Volume 186 Number 2, Issued November 1985 (New York, NY, USA) SEDIVY et al. "Fructose bis-phosphatase of Saccharomyces cerevisiae: cloning disruption and regulation of the FBP1 structural gene" pages 307-319. See pages 307, 318	19,21 2-4,16
X A	The Journal of Biological Chemistry, Volume 260 Number 10, Issued May, 1985 (Bethesda, Maryland, USA) VASSAROTII et al. "Isolation of the fructose-1,6-bisphatase gene of the yeast Schizosaccharomyces pombe" pages 6348-6353. See pages 6348, 6350	19,21 2-4,16

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
06 February 1987	17 FEB 1987
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Karen Maurey

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
A,P	The Journal of Biological Chemistry, Volume 261, Number 9, Issued March, 1986 (Bethesda, Maryland, USA) RITTENHOUSE et al "Amino acid sequence of the phosphorylation site of yeast (Saccharomyces cerevisiae) fructose-1,6-bisphosphatase" pages 3939-3943.	5
A	Chemical Abstracts, Volume 84, Number 17, Issued April, 1976 (Columbus, Ohio, USA) HOOGERHEIDE "Studies on the energy metabolism during the respiratory process by baker's yeast" page 251, abstract number 118248z.	18
X Y	Molecular and Cellular Biology, Volume 4, Number 12, Issued December 1984 (Washington, D.C., USA) HAGUENAUER-TSAPIS et al., "A deletion that includes the signal peptidase cleavage site impairs processing, glycosylation, and secretion of cell surface yeast acid phosphatase" pages 2668-2675 see pages 2668, 2671, 2672, 2674 and Figure 1.	19,20 11-14
A	Gene, Volume 27, Number 1, Issued March, 1984 (Amsterdam, The Netherlands) GOFF et al. "Expression of calf prochymosin in Saccharomyces cerevisiae" pages 35-46, see page 39	2,8,21
T	Biological Abstracts Volume 82, Number 10, Issued November 1986 (Philadelphia, Pennsylvania, USA) VOLKERT et al. "Site-specific recombination promotes plasmid amplification in yeast" abstract number 92596. Cell, 1986, 46(4), 541-550.	10,17,23, 24
X A	Foundation for Biotechnical and Industrial Fermentation Research Volume 1, Issued June, 1983 (Helsinki, Finland) HINNEN et al. "High expression and secretion of foreign proteins in yeast" pages 157-163. See pages 160-162	19,20 11-15

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

Invention I: Claims 1-10, 16, 17, 19, 21-24
Invention II: Claims 11-15, 20
Invention III: Claim 18

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☒ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ATTACHMENT

II. FIELDS SEARCHED:

Keywords: fructose 1,6-bisphosphatase, GAL1 promoter, dinitrophenol, ATP, yeast plasmid, vector cDNA, Szostak, Rogers